Purification and partial characterization of ARG-specific ADP-ribosyltransferase from skeletal muscle microsomal membranes

Jon Eric Peterson
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Purification and partial characterization of ARG-specific ADP-ribosyltransferases from skeletal muscle microsomal membranes

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Iowa State University, 1990
Purification and partial characterization of ARG-specific ADP-ribosyltransferase from Skeletal Muscle Microsomal Membranes

by

Jon Eric Peterson

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HPLC, high-performance liquid chromatography
DEA-BAG, diethylamino(benzylidineamino)guanidine
POE, polyoxyethylene-9-lauryl ether
FAB, fast atom bombardment
LAME, L-arginine methyl ester
HEPES, 4-(2-Hydroxyethyl)-1-piperazineethanesulfonic acid
DABSYL, 4-dimethylaminobenzene-4'-sulfonyl
3-ABA, 3-aminobenzamide agarose
SDS, sodium dodecyl sulfate
PAGE, polyacrylamide gel electrophoresis
DEDICATION

Dedicated to Debbie, Laurie and Krysta, to whom none of this matters.
I. INTRODUCTION

Biological membranes, while responsible for separating compartments, are also involved in the communication of related information. The flow of information depends on the nature of the stimuli and the proteins embedded within the membrane. The proteins either allow transport or propagate a signal between compartments as defined by the system.

Certain hormones, after binding to the exterior of cells, have long been known to alter the intracellular concentrations of cyclic AMP, calcium, cyclic GMP and inositol. These second messengers rapidly diffuse and elicit the cellular response. Signalling of the hormone bound receptor to the effector system that alters intracellular concentrations of second messenger is often times facilitated by a member of the guanyl nucleotide-binding (or G) protein family. This signalling pathway can be disrupted by ADP-ribosylation of the $\alpha$-subunit of the stimulatory or inhibitory G-protein heterotrimeric complex by the enzymatic activities of cholera or pertussis toxin. This post-translational modification can exert varied responses dependent upon which G-protein is modified by which ADP-ribosyltransferase.

The best described, and longest known, effector for the G-protein coupled pathways is adenylyl cyclase, which catalyses the conversion of ATP to cyclic AMP. This second messenger
binds to and dissociates the regulatory subunit of cAMP-dependent protein kinase, thus activating the kinase and evoking a metabolic cascade. The stimulatory G-protein, when stimulated by the occupied receptor, exchanges GTP for GDP, dissociates the GTP bound alpha subunit from the beta/gamma complex which then activates the adenylyl cyclase. The GTP bound alpha subunit, the active form, is rapidly inactivated when the GTP is hydrolyzed by the GTPase activity of the alpha subunit, thus allowing reassociation of the beta/gamma subunits to the alpha with return to the basal state. The inhibitory G-protein, when stimulated by the occupied receptor undergoes a similar dissociation as for the stimulatory G-protein, however the liberated GTP-bound alpha subunit is thought to inhibit adenylyl cyclase. An opposing theory suggests that the alpha subunit of the inhibitory complex is inactive, and that the beta/gamma subunit complex is free to bind basal levels of free stimulatory alpha. At the time of this writing (Spring 1990), there are 16 known G-alpha subunits of 4 different classes, 4 known beta subunits and 2 known gamma subunits. The alpha subunit is considered the most variant and possesses the functionally active GTPase activity. The only assigned roles for the less variant beta/gamma subunits are to regulate the alpha, and provide a membrane anchor. While only a few of the earlier discovered G-proteins effect adenylyl cyclase, the more recently
discovered, and more abundant, alpha subunits are found to affect ion channels.

Adenosine diphosphate-riboyltransferase catalyzes the transfer of ADP-ribose from nicotinamide adenine dinucleotide (NAD) to proteins or acceptor molecules (1,2). Mono-ADP-riboyltransferase, the focus of this research, catalyzes the transfer of one ADP-ribose group from NAD to an acceptor molecule. The poly-ADP-riboyltransferases catalyze the transfer of an ADP-ribose to an acidic amino acid through an O-glycosidic linkage followed by successive β(1-3) polymerizations of several ADP-ribose groups. This class of transferase is normally associated with functions in the nucleus of higher eukaryotes (1,2).

Mono-ADP-riboyltransferase activity has been detected in prokaryote and eukaryote cells. The exotoxins from several prokaryotes possess ADP-riboyltransferase activity with partially defined substrate specificities. The toxin from Vibrio cholera is specific for arginine or small molecular weight guanyl compounds. It has been used extensively as a tool in the elucidation of the stimulatory G-protein coupled effector systems. ADP-ribosylation of the alpha subunit inhibits its GTPase activity, thus persistently activating adenylyl cyclase (1). The toxin from Bordetella pertussis ADP-ribosylates a cysteine residue of the inhibitory G-protein alpha subunit, which dissociates the G-protein heterotrimer
from the receptor, thus interfering with the receptor coupled dissociation of the alpha subunit. The net effect of ADP-ribosylation by either toxin is persistent activation of adenyl cyclase (1,2). The toxin from Corynebacterium diphtheria modifies the dipthamide residue of the ribosomal elongation factor 2, thus shutting of protein biosynthesis (1,2). The toxin from Clostridium botulinum modifies asparagine of rho small molecular weight GTP-binding proteins, with a presently unknown physiological effect (3).

Several types of animal cells have also been found to possess endogenous mono-ADP-ribosyltransferase activities (1,2). The physiological significance of an endogenous enzymatic activity resembling pathogenic toxins has puzzled investigators for more than a decade. It is suspected that the ADP-ribosyltransferase plays a role in signal transductional processes; however, the regulation and compartmentalization must be very strict to preempt pathological cell death.

Moss and associates have reported the purification and characterization of four classes of mono-ADP-ribosyltransferase from turkey erythrocyte. Types A (4) and B (5) were purified from soluble fractions, type C (6) was purified from the peripherally associated membrane fraction, and type D (7) was purified from the nuclear fraction. Most of the identified endogenous mono-ADP-ribosyltransferases catalyze the
formation of N-glycosidic bonds between ADP-ribose and arginine (1,2). However, a cysteine specific ADP-ribosyltransferase activity was recently identified and purified from human erythrocytes (8).

Several tissues have been reported to possess an enzyme catalyzing the reverse reaction, ADP-ribosylhydrolase (9,10,11), which implies a physiological significance. Studies of ADP-ribosyltransferase and ADP-ribosylhydrolase activities in *Rhodospirillum rubrum* (12) delineated an elegant regulation of the catalytic Fe iron protein center of nitrogenase.

ADP-ribosyltransferase seems almost ubiquitous in its tissue type distribution. The activity has been detected in turkey erythrocyte, rat liver, *Xenopus laevis* liver, lung, muscle, heart and erythrocyte, bovine thyroid, bovine, porcine and rabbit skeletal muscle, *Torpedo* synaptosomes, rat Islets of Langerhans, adipocyte plasma membranes (1) and human platelets (13).

Soman and associates detected (14) and partially characterized the endogenous mono-ADP-ribosyltransferase activity in skeletal muscle sarcoplasmic reticulum of rabbit and sarcolemma of pig (15).

The purification of the integral membrane mono-ADP-ribosyltransferase has been of primary interest to characterize and eventually establish a metabolic role for
this interesting enzyme.

This dissertation describes the first purification of the mammalian integral membrane mono-ADP-ribosyltransferase and its partial characterization. The purification is a rapid method that requires only 3 days from start to finish. Positive identification of the mono-ADP-ribosyltransferase was confirmed by a characterized zymographic localization and HPLC assay of gel slice incubations.
II. MATERIALS AND METHODS

A. Materials Used

Frozen mature rabbit skeletal muscle was purchased from Pel-Freeze Biologics, DE-52 was purchased from Whatman, Toyopearl DEAE was purchased from Supelco, Triton X-100 was obtained from Boehringer-Mannheim, the silver stain kit, SEC-250 size exclusion column and molecular weight markers were from Bio-Rad. The molecular weight markers had resolved masses of: phosphorylase b, 97,400 Da; bovine serum albumin, 66,200 Da; ovalbumin, 42,699 Da; and carbonic anhydrase, 31,000 Da. BCA Protein Assay kits (standard and micro) and OPA reagent were purchased from Pierce Chemical Company, 3-methoxybenzamide was purchased from Aldrich, [P-32] NAD was purchased from ICN, Centricon 30 microconcentrators were purchased from The Amicon Company. Remaining reagents and chemicals were obtained from the Sigma Chemical Company.

B. Methods

Arginine specific mono-ADP-ribosyltransferase was determined by the use of three assays.

1. L-arginine methyl ester assay

An arginine methyl ester HPLC assay using precolumn OPA derivatization and postcolumn fluorescence detection was as
described in (16). Briefly, the reaction mixture contained 50 mM potassium phosphate, pH 7.0, 10 mM L-arginine methyl ester (LAME), and 2 mM NAD in a total volume of 100 uL. Reactions were initiated by addition of enzyme at a linear dilution and incubated at 30°C for the indicated amount of time in the linear range. The HPLC system consisted of two Beckman model 110A pumps with a 421 gradient programmer. Solvent A = 50 mM potassium phosphate, pH 4.0, and solvent B = 5% THF/95% MeOH. An aliquot of the reaction mixture was derivatized with an equal volume of OPA/β-mercaptoethanol reagent for 1 minute and injected onto a C-8 reversed phase column at a flow rate of 1.5 ml/min. The gradient conditions were 5% - 100% B in 5 min. OPA products were detected with a Kratos FS950 Fluorometer with an excitation wavelength of 365 nm and an emission wavelength of 418 nm. Total analysis time for each sample was 15 min with ADP-ribosylated LAME eluting at 3.9 min. Conversion rates were determined by peak height analysis and comparison made to ADP-ribosylated LAME standard curves. Typically, the assay calculations included the dilution factors multiplied by 79 pmole ADP-ribosylated LAME per mm peak height. In all assays, the values represent those obtained in the linear dilution range and at linear incubation times. Bovine serum albumin was added (0.05 mg/ml) to stabilize the enzyme in those fractions with dilute protein concentrations.
2. **Guanylhydrazone assay**

The guanylhydrazone diethylamino-(benzylidene-amino)guanidine (DEA-BAG) HPLC assay was as described previously ([15,17,18]) with some modifications. The enzyme was incubated in 2 mM DEA-BAG, 10 mM NAD, 100 mM phosphate buffer for 1 h at 30°C. The reactions were stopped with an equal volume of 20% trichloroacetic acid, centrifuged and stored for up to 2 weeks at -20°C. The HPLC system consisted of two Beckman pumps (models 100A and 110A) with a 420 gradient programmer, a Beckman 165 variable wavelength detector and a Nelson Analytical 760 Series Interface to an IBM PC for data analysis. Solvent A = 0.2 M triethylammonium acetate, 0.1 M sodium perchlorate, pH 6.0, and solvent B = methanol. An aliquot of the stopped reaction mixture was injected onto a C18 (5 μm) reverse-phase column at a flow rate of 1 ml/min. Gradient conditions were 0 - 50% B in 1 min and held at 50% for 25 min. Total analysis time was 25 min, with the anomeric ADP-ribosylated DEA-BAG products eluting at 16 and 17 min. Conversion rates were determined by integration. One unit was defined as 1 pmole DEA-BAG converted per min.

3. **Zymographic in situ gel assay**

The zymographic polyacrylamide in situ gel assay was essentially as described previously ([19]) but with the inclusion of 1 mM ADP-ribose at a 10-fold molar excess to NAD
to eliminate nonenzymatic reactions. Prior to electrophoresis, protein samples were precipitated from detergent and lipid contaminants as described previously (20) to prevent smearing during electrophoresis. Briefly, 30 volumes ethanol:acetone (1:1) were mixed with 50 \( \mu l \) sample with variable protein concentrations, chilled to -20°C for at least 1 h, centrifuged, and the supernatants discarded. Residual solvent was removed in a Speed-Vac. Our studies displayed 96% recovery of protein (data not shown). Delipidated samples were incubated in 1% sodium dodecyl sulfate, 10% glycerol, 0.005% bromophenyl blue and 50 mM Tris-HCl, pH 6.8, for 5 min at room temperature. Samples were electrophoresed according to (21) in 10% acrylamide mini gels (0.75 x 60 x 80 mm) for 30 min at 200 V. Electrophoretic times greater than 1 h destroyed enzymatic activity. The ADP-ribosyltransferase was renatured by incubating the gel in 20 volumes of 2.5% Triton X-100 for two cycles of 0.5 h and one cycle in water for 0.5 h. The gel was transferred to a heat-sealing plastic bag and incubated in two volumes 100 mM phosphate, pH 7.5, 1 mM dithiothreitol, 400 mM NaCl, 2 mg/ml histone (Sigma, Type VIIIs), 1 mM ADP-ribose, and 100 uM [P-32] NAD (100 cpm/pmol) for up to 4 h at room temperature. The reactions were stopped in 50% TCA, 0.1% Coomassie blue. The gels were destained in 7% acetic acid/30% methanol until the background was cleared. The gels were also silver-stained.
according to the BioRad instructions, dried and exposed to X-Omat autoradiography film in the presence of intensifying screens. The molecular mass determination for the enzyme was made by comparison of the relative mobility of the enzyme with those of the molecular weight markers on graph of the log molecular mass versus relative mobility. Alignment of the bands with the appropriate size was confirmed by running carbonic anhydrase in an adjacent lane.

4. Buffers used

Homogenization buffer (Buffer A) was 10 mM histidine, 10% sucrose, 1 mM benzamidine, 1 mM EDTA, 1 mM iodoacetamide, 77 nM aprotinin, 0.7 μM pepstatin, 1.1 μM leupeptin, 0.23 mM phenylmethylsulfonylfluoride (pH 7.0). The KCl wash buffer (Buffer B) was 0.6 M KCl, 10 mM histidine, 1 mM benzamidine, 1 mM EDTA, 1 mM iodoacetamide, 77 nM aprotinin, 0.7 μM pepstatin, 1.1 μM leupeptin, 0.23 mM phenylmethylsulfonylfluoride (pH 7.0). The propylene glycol wash buffer (Buffer C) was 20% propylene glycol, 50 mM Tris, 0.1% Triton X-100, 1 mM benzamidine, 1 mM EDTA, 1 mM iodoacetamide, 77 nM aprotinin, 0.7 μM pepstatin, 1.1 μM leupeptin, 0.23 mM phenylmethylsulfonylfluoride (pH 7.5). DE-52 buffer (Buffer D) was 10 mM phosphate, 10% glycerol, 0.05% sodium deoxycholate, 1 mM benzamidine, 1 mM EDTA (pH 7.5). Affinity buffer (Buffer E) was 50 mM Tris, 0.5% polyoxyethylene-9-lauryl ether, 0.2 M
NaCl (pH 7.5). Size exclusion chromatography buffer was 50% ethylene glycol, 100 mM phosphate, pH 7.0.

5. Preparation of skeletal muscle microsomes

Microsomal membrane was prepared by a method modified from previously described methods (22,23). All steps were performed at 4°C. Mature rabbit skeletal muscle (0.5 kg) was thawed, passed through a meat grinder, and homogenized in 1.4 liters of Buffer A at high speed for 1 min in a commercial size Waring Blendor. The homogenate was centrifuged at 15,000 x g for 0.5 h, filtered through glass wool and centrifuged at 100,000 x g for 2 h. The pellet was resuspended and homogenized with a hand held tissue grinder in Buffer B (150 ml), stirred for 1 h, and centrifuged at 100,000 x g for 1 h. The pellet was resuspended and homogenized in Buffer C (50 ml), stirred for 1.5 h and centrifuged at 105,000 x g for 3 h. The final pellet was resuspended in Buffer A (30 ml or ≥ 10 mg/ml) and called 100K x g pellet. Protein concentration was determined prior to the solubilization step.

6. Synthesis of inhibitor affinity column

3-aminobenzamide agarose was prepared by a modification of a described method (24). 3-aminobenzamide was succinylated with succinic acid anhydride with a 95% yield after hot ethanol washes. Positive FAB mass spectrometry confirmed the
presence of one major molecular ion of 237.1, which conforms to the 236 Da molecular weight of succinylated 3-aminobenzamide. The molar extinction coefficient at 318 nm was estimated to be 1247 cm⁻¹ M⁻¹. A linker 3,3'-iminobispropylamine was coupled to CNBr activated agarose at pH 10-11 in 100 mM phosphate and fluorescamine analysis (25) determined the spacer arm concentration to be about 48 mM. Final coupling was achieved by dissolving 1 g succinylated 3-aminobenzamide (4 mmole) in 20 ml dimethylformamide followed by the addition of 2.5 ml derivatized agarose in 5 ml H₂O and 0.769 g (4 mmole) 1-ethyl-3-(3-methylaminopropyl)carbodiimide (26) dissolved in 5 ml 0.5 M phosphate at pH 5. Spectral scanning of a 10% suspension of the derivatized agarose in 10% glycerol, 10 mM phosphate buffer, pH 7.5, indicated a maxima at 288 nm and a ligand density of 12 mM.

7. **Time course of zymographic reaction**

Muscle mono-ADP-ribosyltransferase, 100 µg (concanavalin A eluate, 293,000 U/mg) was precipitated with 30 volumes ethanol:acetone, (1:1), centrifuged and the pellet lyophilized. The pellet was dissolved in 130 µl 1% SDS, 50% glycerol, 50 mM Tris, 0.005% bromophenyl blue, pH 6.8 and incubated for 5 min at 30°C. Fifteen µl was added to each of 8 wells of an analytical mini-gel (10% acrylamide, 0.75mm X 60mm X 80mm) and electrophoresed for 30 min at 200 V. The gel
was renatured in 2.5 % Triton X-100 as described previously and 8 gel slices were removed from the 38 - 39 kDa region of each of the lanes. The gel slices were equilibrated for 5 min in 2 ml zymographic buffer containing all of the components except NAD. At time zero, 100 μM NAD [P-32], 100 cpm/pmole, was added and the incubation carried out at room temperature. At times 0, 15, 30, 90, 120, 180 and 240 min, a gel slice was transferred to a labeled vial containing 50 % TCA 0.1 % coomassie blue, fixed for 1 h followed by extensive washing with 30 % Methanol, 7 % acetic acid un-til the gel slices were clarified. Cerenkov counting of the slices was performed and the values tabulated.

8. Hydroxylamine sensitivity

Hydroxylamine sensitivity reactions were done similar to those described by Hsia et al. (27). To 250 μg of mono-ADP-ribosyltransferase (concanavalin A elute, 170,000 U/mg) was added 5 ml ethanol:acetone (1:1). The mixture was cooled, centrifuged, the supernatant decanted and the precipitate lyophilized. This delipidated and detergent free precipitate accounted for about 96% of the membrane protein. The dried protein was dissolved in 200 μl 1% SDS, 10 % glycerol, 50 mM Tris, 0.005% bromophenyl blue (pH 6.8), incubated for 5 min and applied to a preparative mini-gel (10 % acrylamide, 1mm x 60mm x 80 mm), electrophoresed and carried through the
zymographic reaction as described previously except with 1000
cpm/pmole [P-32] NAD. The autoADP-ribosylated monoADP-
ribosyltransferase was electroeluted from the 38 - 39 kDa
region of the gel and concentrated with a centricon 30 to 100
μl. Protein recovery was 25 μg (10 %). The sample was
aliquoted into two tubes, each containing 10 μg protein (250
pmole) and about 2000 cpm [P-32] (20 pmole ADP-ribose),
precipitated with 30 volumes ethanol:acetone (1:1) and dried.
To each tube was added 250 μl of either 0.5 M hydroxylamine,
1 % SDS, 50 mM HEPES, pH 7.5, or 0.5M NaCl, 1% SDS, 50 mM
HEPES, pH 7.5, and incubated at 37oC. At times 0, 60, 90 and
120 minutes, 50 μl (2 μg) was removed from the incubations and
added to 200 μl 20% cold TCA and the precipitates collected by
centrifugation. The pellets were dissolved in 2% SDS, 50 %
glycerol, 50 mM Tris, 0.005 % bromophenyl blue (pH 6.8),
applied to an analytical mini-gel (10% acrylamide, 0.75mm x
60mm x 80mm), electrophoresed, fixed with 50 % TCA, 0.1 %
coomassie, washed with 30 % methanol, 7 % acetic acid, dried
and exposed to X-Omat film in the presence of intensifying
screens. Several densitometry scans of each lane provided a
unitless value for graphical representation. Cerenkov
counting of the pellets post hydroxylamine or NaCl and prior
to electrophoresis (data not shown) gave values closely
resembling those obtained from densitometry.
9. **3-aminobenzamidine inhibition of zymography**

Muscle mono-ADP-ribosyltransferase, 60 μg (concanavalin A eluate, 293,000 U/mg) was precipitated with 30 volumes ethanol:acetone, (1:1), centrifuged and the pellet lyophilized. The dry pellet was dissolved in 80 μl 1% SDS, 50% glycerol, 50 mM Tris, 0.005% bromophenyl blue, pH 6.8 and incubated for 5 min at 30°C. Fifteen μl was added to each of 5 wells of an analytical mini-gel (10% acrylamide, 0.75mm X 60mm X 80mm) and electrophoresed for 30 min at 200 V. After electrophoresis, the gel was renatured in 2.5% Triton X-100 as described previously. Five gel slices were removed from the 38-39 kDa region of each of the lanes and one slice transferred to scintillation vials each containing 2 ml zymographic reaction components as described previously and 0 to 20 mM 3-aminobenzamidine. The reactions were allowed to proceed for 4 h, followed by fixing in 50% TCA, 0.1% coomassie blue and washing with 30% methanol, 7% acetic acid to clarity and Cerenkov counting. A Dixon-Webb plot was prepared by plotting 1/cpm P-32 labeled 38.5 kDa protein vs inhibitor concentration.

10. **Histone stimulation of zymographic reaction**

Muscle mono-ADP-ribosyltransferase, 100 μg (concanavalin A eluate, 293,000 U/mg) was precipitated with 30 volumes ethanol:acetone, (1:1), centrifuged and the pellet
lyophilized. The dry pellet was dissolved in 130 μl 1% SDS, 50 % glycerol, 0.005 % bromophenyl blue, pH 6.8 and incubated for 5 min at 30°C. Fifteen μl was added to each of 8 wells of an analytical mini-gel (10 % acrylamide, 0.75mm X 60mm X 80mm) and electrophoresed for 30 min at 200 V. The gel was renatured in 2.5 % Triton X-100 as described previously and 8 gel slices were removed from the 38 - 39 kDa region of each of the lanes. The slices were equilibrated for 5 min in scintillation vials containing 2 ml zymographic reaction components with 0, 10, 20, 40, 80, 160, 320 and 480 μg/ml histone (Sigma, type VIIIs). The reactions were allowed to proceed for 4 h, followed by fixing, washing to clarity and Cerenkov counting.

11. Determination of auto-ADP-ribosylation

Muscle mono-ADP-ribosyltransferase, 60 μg (concanavalin A eluate, 293,000 U/mg) was precipitated with 30 volumes ethanol:acetone, (1:1), centrifuged and the pellet lyophilized. The dry pellet was dissolved in 80 μl 1% SDS, 50 % glycerol, 0.005 % bromophenyl blue, pH 6.8 and incubated for 5 min at 30°C. Fifteen μl was added to each of 5 wells of an analytical mini-gel (10 % acrylamide, 0.75mm X 60mm X 80mm) and electrophoresed for 30 min at 200 V. After electrophoresis, the gel was renatured in 2.5 % Triton X-100 as described previously. Five gel slices were removed from the
38 - 39 kDa region of each of the lanes and transferred to a scintillation vial with the zymographic reaction components including 2 mg/ml histone (Sigma type VIIIs) and allowed to react for 4 h, followed by fixing in 50 % TCA, 0.1 % coomassie blue and washing to clarity in 30 % methanol, 7 % acetic acid. The gel slices were electroeluted and the sample concentrated. Eighteen micro-grams of protein was recovered in the electroeluted sample. To an analytical mini-gel (0.75mm x 60mm x 80mm) was applied 6 µg electroeluted protein and 5 µg histone (Sigma, type VIIIs) in separate lanes dissolved in 1 % SDS, 50 % glycerol, 50 mM Tris, 0.005 % bromophenyl blue, pH 6.8. The samples were electrophoresed for 30 min at 200 V, fixed in 50 % TCA, 0.1 % coomassie blue and destained in 30 % methanol, 7 % acetic acid, dried and exposed to X-OMAT film with intensifying screens.

12. **Comparison of cholera toxin and muscle transferase**

Cholera toxin A Sigma, 6 and 12 µg, 22,000 U/mg) was diluted in sample buffer to provide final concentrations of 1 µg/µl toxin, 1% SDS, 50 % glycerol, 50 mM Tris, 0.005 % bromophenyl blue pH 6.8 with or without 2.5 % β-mercaptoethanol. Muscle mono-ADP-ribosyltransferase (concanavalin A eluate, 40 µg, 150,000 U/mg) was precipitated with ethanol:acetone (1:1), centrifuged, the pellet lyophilized and dissolved in sample buffer to provide final
concentrations of 2 μg protein/μl, 1% SDS, 50% glycerol, 0.005% bromophenyl blue, pH 6.8, with or without 2.5% β-mercaptoethanol. After 5 min preincubation, the samples were electrophoresed and carried through the zymographic assay as described previously.

13. Gel-slice incubation with guanylhydrazone

Ten μl Mono-ADP-ribosyltransferase (concanavalin A eluate, 5.4 μg protein, 172,000 U/ml, 320,000 U/mg) was precipitated with 30 volumes ethanol:acetone (1:1), centrifuged, the supernatant decanted and the pellet lyophilized. Twenty μl of 1% SDS, 50 mM Tris, 50% glycerol, 0.005% bromophenyl blue, pH 6.8, was added and the sample electrophoresed in a mini-gel (10% acrylamide, 0.75 mm X 60 mm X 80 mm) for 30 min at 200 V with prestained molecular weight markers as reference. At the end of the electrophoresis, the gel was renatured by incubation with 20 volumes 2.5% Triton X-100 for two cycles of 30 min each followed by one cycle in water for 30 min. The gel slices from the 38,500 Da region were cut from the gel and diced (not minced) to 0.75 mm cubes with a sharp razor, and transferred to an assay mixture containing 100 mM potassium phosphate, pH 7.5, 10 mM NAD, 2 mM DEA-BAG in a final volume of 300 μl. Incubations were conducted at 30°C with frequent agitation. At times 0, 2 and 4 h, 90 μl aliquots were removed and added to 150 μl 20% TCA. The stopped reactions were
analyzed for ADP-ribosylated DEA-BAG in the HPLC assay as previously described.

14. **ADP-ribosylhydrolase reaction**

ADP-ribosylhydrolase from rat skeletal muscle, was partially purified from fresh rat skeletal muscle as previously described (28). Muscle (375 g) was homogenized in 1 L buffer P (20 mM potassium phosphate, 1 mM EDTA pH 7.5), centrifuged at 1500 x g for 30 min followed by centrifugation of the glass wool filtered supernatant at 45,000 x g for 60 min. The supernatant was (800 ml) was applied to a DE-52 column, equilibrated in buffer P and washed with the same buffer. The ADP-ribosylhydrolase activity was eluted at 0.5 M NaCl in buffer P (440 ml) and applied to a phenyl-Sepharose Cl-4B column equilibrated with 0.5 M NaCl in buffer P, and washed with the same buffer. ADP-ribosylhydrolase activity was eluted with 50 % propylene glycol in buffer P (170 ml) and applied to a hydroxylapatite column followed by elution with a linear gradient of 20 to 500 mM potassium phosphate in 20 % propylene glycol, 1 mM EDTA, pH 7.5. The eluant containing ADP-ribosylhydrolase activity (102 ml) was desalted on a Sephadex G-25 column and applied to a DE-52 column which was equilibrated with buffer P. The ADP-ribosylhydrolase activity was eluted in a linear gradient of 0 to 300 mM NaCl in buffer P, dialyzed against buffer P and used for enzymatic studies.
(74 ml, 79 fold purification, 2886 U/ml, 493 U/mg). One unit of ADP-ribosylhydrolase was defined as that amount of enzyme required to convert 1 nmole of ADP-ribose DABSYL-arginine methyl ester per min in a HPLC assay (28). Three reactions were carried out containing ADP-ribosyltransferase and ADP-ribosylhydrolase, ADP-ribosyltransferase alone or ADP-ribosylhydrolase alone. ADP-ribosyltransferase, 1600 U (Con A eluant, 12 µg, 160,000 U/ml, 133,000 U/mg) was incubated with 2.5 U ADP-ribosylhydrolase (DE-52 eluant II, 5 µg 74 U/ml, 493 U/mg). The incubations were conducted in 10 mM phosphate buffer, 15 mM MgCl₂, and 5 mM dithiothreitol in a total volume of 50 µl for 1 h at 30°C. Reactions were stopped by the addition of 1.5 ml ethanol:acetone (1:1), chilled, centrifuged, the supernatants discarded and the pellets lyophilized. The protein pellets were dissolved in 1 % SDS, 50 % glycerol, 50 mM Tris, 0.005 % bromophenyl blue, pH 6.8, electrophoresed and carried through the zymographic assay as previously described. The gel was dried, exposed to X-OMAT film in the presence of intensifying screens. After exposure to film, the bands were cut out of the gel and counted by Cerenkov spectrometry.
III. RESULTS
A. Purification

1. Solubilization with sodium deoxycholate

Sodium deoxycholate (DOC) released an abundant amount of ADP-ribosyltransferase activity into the soluble phase. Several concentrations of deoxycholate were analyzed to determine the optimum concentration for ADP-ribosyltransferase solubilization (data not shown). The concentration of 0.3\% sodium deoxycholate (w/v at a protein concentration of 10 mg/ml) provided the maximum amount of solubilization without rapid denaturation. To the 100K x g pellet, enough sodium deoxycholate was dissolved in buffer A to provide a 0.3 \% (w/v) concentration of detergent with a 10 mg/ml final concentration of protein. The mixture was stirred at 4°C for 0.5 h and centrifuged at 100,000 x g for 1.5 h at 4°C. The opalescent supernatant was removed with a pipet and the light sediment and pellet were discarded. Typical preps yielded 60 - 80 ml containing 6 mg/ml protein. Due to the rapid half-life of the enzyme in 0.3\% DOC (about 1 day at 4°C) the DE-52 step was done immediately after solubilization.

2. DE-52 cellulose chromatography

A panel of columns was screened to assess retention and elution of the deoxycholate solubilized ADP-ribosyltransferase activity. The activity did not bind to cation exchangers but
was retained on DEAE sepharose and Toyopearl DEAE and DE-52 cellulose anion exchangers as measured by the lack of or low levels of activity in the fall-through fractions. The yields of enzyme eluted in gradients from 0 to 1 M KCl were ≈ 4% for DEAE cellulose, ≈ 2% for Toyopearl DEAE and ≈ 60 - 70% for DE-52. Because of the anionic nature of deoxycholate, a more complete elution from the anion exchangers was explored by dialyzing the deoxycholate extract with several nonionic detergents preceding chromatography. In all instances, including DE-52, all or most of the activity was found in the fall-through fractions (data not shown). Loading of DE-52 in the presence of DOC and switching to Lubrol PX resulted in immediate nonselective elution of the monoADP-ribosyltransferase activity. Concentrations as low as 0.05% DOC were sufficient to retain the activity on the anion exchanger. DE-52 microgranular anion exchange cellulose (Whatman), was converted to the chloride form and used once, then discarded. The DE-52 was equilibrated and degassed in buffer D before pouring, packing, and washing of the column with 5 additional volumes of buffer D.

The column was run at room temperature by loading approximately 160 mg 0.3% DOC solubilized membrane protein in 26 ml buffer A onto a 2.5 x 16 cm (80 ml) DE-52 column at a flow rate of 2 ml/min and eluted with 0 - 1 M KCl in buffer D over 500 ml (figure 1). A positive flow rate was maintained
at 2 ml/min with a peristaltic pump. The protein elution profile was monitored by an ISCO in-line UV detector. Sixty fractions of 8 ml each were assayed for ADP-ribosyltransferase activity and the pool made with those fractions containing the highest ADP-ribosyltransferase activity. Assays were done with 20 ul of each fraction, 10 mM LAME, 2 mM NAD and 50 mM potassium phosphate, pH 7.0. Typical preps yielded 40 - 50 ml containing 0.5 mg/ml protein. Protein loads of solubilized membrane protein above 2 mg per ml column volume overloaded the column and greater than 50% of the activity was found in the fall-through fractions.

Figure 1 shows the elution profile of ADP-ribosyltransferase activity from the DE-52 column. The enzymatic activity is represented by the dashed peak and a conservative pool of the activity was made from fractions with specific activities over 1000 U/ml. This column has been repeated up to 15 times with essentially the same profile at room temperature. If the DE-52 column step was performed at 4°C, resolution was lost, and the activity was spread over many fractions.

3. **Concanavalin A agarose chromatography**

Concanavalin A agarose (Sigma Chemical Co.) was found to bind 100% of the mono-ADP-ribosyltransferase activity from the DE-52 pool. The presence of 0.05% DOC did not interfere with concanavalin A binding.
Figure 1. DE-52 chromatography profile. The ADP-ribosyl-transferase activity elution is indicated by the dashed line (---) in Units/ml where 1 U is defined as that amount of enzyme required to convert 1 pmole LAME per min. The KCl gradient is shown with (----). Details are given in "Methods."
Wheat germ agglutinin did not bind the activity (data not shown). Figure 2 shows the elution profile of a 2 ml concanavalin A agarose column loaded with a 50 ml pool from the DE-52 pool, followed by washing with 20 ml buffer E (W) followed by elution (E) with 10 ml 0.3 M methylmannopyranoside in buffer E. One ml fractions were assayed for protein concentration and ADP-ribosyltransferase activity by the LAME HPLC assay. Dilution factors of 10 for the fall through and washes and 50 for the eluant were used for screening the column and pooling the activity. Typically, the combined pools from two DE-52 column runs (80 - 100 ml) were passed twice through a 1.5 X 1.7 cm (3 ml) concanavalin A agarose column at a flow rate of 2 ml/min. No ADP-ribosyltransferase activity was found in the fall-through fraction. The column was washed with 10 volumes buffer E and equilibrated in two volumes elution buffer (0.3M methylmannopyranoside in buffer E) for 12 h at 4°C prior to elution. Elution was completed with an additional 3 volumes of elution buffer.

The enzymatic activity in elution buffer was reduced in methyl-mannopyranoside concentration to less than 0.01 M by concentration in Centricon 30 microconcentrators to a volume of 0.5 ml, followed by subsequent addition and concentration in buffer E to a volume of about 1 ml. This was necessary to reduce the interference in the protein assay and subsequent chromatography step. At this stage of the purification, the
enzyme was stored at -20°C for up to 4 weeks without any appreciable loss of activity. The concanavalin A agarose was washed and recycled with either 20 volumes Buffer E alone or Buffer E containing 0.3 M methylmannopyranoside for up to 10 times.

![Concanavalin A agarose chromatography profile](image)

**Figure 2.** Concanavalin A agarose chromatography profile. The ADP-ribosyltransferase activity elution is indicated by the dashed line (---) in Units/ml where 1 U is that amount of enzyme required to convert 1 pmole LAME per min. Details are given in "Methods."
4. **3-aminobenzamide agarose chromatography**

The concanavalin A agarose eluate, when applied to the 3-aminobenzamide agarose column resolved two molecular weight forms of the monoADP-ribosyltransferase as described later in the "Results" section. Typically 1.0 to 1.5 ml concanavalin A agarose eluant fractions containing 600,000 -1,000,000 U were applied to 2 ml 3-aminobenzamide agarose column equilibrated in buffer E. The con A eluate was allowed to equilibrate with the column overnight at 4 C. After eluting the unbound fraction (3ABA fall through), the column was rinsed with 5 volumes buffer E. The column was then equilibrated with 25 mM 3-methoxybenzamide for 1 h prior to elution of the activity. One ml fractions were assayed for protein using the micro BCA (Pierce) kit and LAME enzymatic activity determined at a dilution factor of 100. Eighty % of the activity was found in the fall through fraction, 15% was eluted with 25 mM 3-methoxybenzamide in buffer E, and the remainder of the activity was recovered in the regeneration step with 2 M KCl in buffer E. The 3-methoxybenzamide elution ligand, a known inhibitor of ADP-ribosyltransferase was removed by successive dilution and concentration in buffer E with centricon 30 microconcentrators (Amicon). The active enzyme eluted with 3-meth-oxybenzamide was > 95 % homogeneous as determined by silver-stained electrophoretic gels.

Figure 3 is a 3-aminobenzamide column profile.
Concanavalin A agarose eluant, 1.3 ml (285,000 U) was applied to a 2 ml 3-aminobenzamide column and reapplied a total of 4 times. The column was washed (W) with 10 ml buffer E followed by elution with 10 ml 25 mM 3-methoxybenzamide (E) in buffer E and 10 ml regeneration buffer (R) 2 M KCl in buffer E. Close to 80% of the mono-ADP-ribosyltransferase activity in the concanavalin A eluant did not bind to the 3-aminobenzamide column. That which did bind was subsequently eluted, characterized and described later.

Figure 3. 3-aminobenzamide agarose column profile. The eluted ADP-ribosyltransferase is depicted with the dashed line (---). The wash step is shown with (W), the elution step (E) and the regeneration step with (R). Details are given in the text.
If the ligand density of the 3-aminobenzamide on the agarose was less than 6 mM, binding of the α form did not occur. To assure maximal binding, all of the coupling reactions must be done at buffered pH ranges as described in "Methods."

5. Size exclusion chromatography

The enzyme activity found in the 3-aminobenzamide fall through fraction was exchanged into 50% ethylene glycol, 100 mM phosphate, pH 7.0 by spin dialysis (centricon 30) and up to 100 ug protein in 200 ul buffer was injected into a 600 x 7.5 mm SEC-250 size exclusion HPLC column. The equilibration and isocratic running buffer was 50% ethylene glycol, 100 mM phosphate, pH 7.0. At a flow rate of 0.25 ml/min, 1 ml fractions were collected and assayed with the LAME-HPLC assay at a dilution factor of 10.

Figure 4 is a SEC-250 column profile. The first peak of activity (12 - 15 ml) is the break-through fraction containing a mixture of proteins. The peak of activity eluting at 20 - 21 ml was found to contain nearly pure ADP-ribosyltransferase with a silver-staining band at 38,500 Da and a specific activity of 6 um/min/mg. All fractions with activity were pooled separately, concentrated in Centricon 30 microconcentrators and frozen without loss of appreciable activity for up to 4 weeks prior to use.
Figure 4. **SEC-250 size exclusion column profile.** The elution of ADP-ribosyltransferase activity is depicted with the dashed line. The activity eluting at 20 - 21 ml was the highly purified fraction.
6. **Quantitative summary of the purification**

Table 1 is a quantitative summary of the steps of purification. The recovery of the pure α-form of the enzyme was 2.0% with about 200-fold purification. The specific activity of ADP-ribosyltransferase type α (1,900,000 U/mg) was about 82 times that for cholera toxin A, 23,100 U/mg. The β-form of ADP-ribosyltransferase was purified to > 80% homogeneity with a 2.1% yield to a specific activity of about 6,000,000 U/mg, which is about 3 times higher than the specific activity of the α-form of the enzyme. The purification values are relative to the 100 K x g microsomal membrane fraction because of the consistent linear activity with activity assays at various dilutions and times. While activity is detectable in the 15 K x g supernatant, there appear to be factors that interfere with the assay. Generally, the activity in the 100 K x g pellet (microsome) had 10 - 70 fold higher specific activities than the 15 K x g supernatant.

Units were expressed as pmol LAME converted per min as determined with the LAME HPLC assay as described in "Experimental Methods." All initial velocities were determined at linear dilution and time. Incubation assays included 10 mM LAME, 5 mM NAD, 0.1 mg/ml bovine serum albumin, 50 mM phosphate pH 7.0 and the following enzyme fraction protein concentrations.
### TABLE 1. Quantitative summary of the purification

<table>
<thead>
<tr>
<th>STEP&lt;sup&gt;a&lt;/sup&gt;</th>
<th>VOLUME (ml)</th>
<th>U/ML x10&lt;sup&gt;-6&lt;/sup&gt;</th>
<th>UNITS (total)</th>
<th>%YIELD</th>
<th>PROTEIN (mg/ml) x10&lt;sup&gt;-6&lt;/sup&gt;</th>
<th>PURIF X-fold</th>
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</thead>
<tbody>
<tr>
<td>1) 15K × g</td>
<td>1040</td>
<td>0.002</td>
<td>2.28</td>
<td>-</td>
<td>17</td>
<td>0.00013</td>
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<tr>
<td>2) 100K × g</td>
<td>24</td>
<td>0.457</td>
<td>10.96</td>
<td>100</td>
<td>49</td>
<td>0.0093</td>
</tr>
<tr>
<td>3) DOC sol</td>
<td>104</td>
<td>0.105</td>
<td>10.90</td>
<td>99</td>
<td>4</td>
<td>0.026</td>
</tr>
<tr>
<td>4) DE-52</td>
<td>86</td>
<td>0.0516</td>
<td>4.44</td>
<td>40</td>
<td>0.5</td>
<td>0.103</td>
</tr>
<tr>
<td>5) Con A</td>
<td>2.4</td>
<td>1.58</td>
<td>3.8</td>
<td>35</td>
<td>0.31</td>
<td>5.1</td>
</tr>
<tr>
<td>6) type α</td>
<td>0.62</td>
<td>0.389</td>
<td>0.23</td>
<td>2</td>
<td>0.2</td>
<td>1.94</td>
</tr>
<tr>
<td>7) 3ABA FT</td>
<td>1.34</td>
<td>1.7</td>
<td>2.28</td>
<td>21</td>
<td>0.3</td>
<td>5.6</td>
</tr>
<tr>
<td>8) type β</td>
<td>0.20</td>
<td>1.2</td>
<td>0.2</td>
<td>2</td>
<td>0.2</td>
<td>6.0</td>
</tr>
</tbody>
</table>

<sup>a</sup> Step 1, 15,000 × g filtered supernatant, 3.4 mg/ml; Step 2, 100,000 × g pellet suspension, 1.2 mg/ml; Step 3, 0.3% deoxycholate solubilized enzyme, 0.10 mg/ml; Step 4, DE-52 pool, 0.013 mg/ml; Step 5, concanavalin A agarose eluant, 3.0 μg/ml; Step 6, 3-aminobenzamide agarose eluant (type α), 3 μg/ml; Step 7, 3-aminobenzamide agarose fall through, 1.5 μg/ml; Step 8, size exclusion HPLC fraction (20 - 21 ml) (type β), 1 μg/ml.

<sup>b</sup> Total units lower than that for 100 K × g attributed to interference in the assay with the 15 K × g supernatant.
7. **Qualitative summary of the purification**

The SDS-PAGE protein profiles of the various stages of the purification are shown in Figure 5. Lane 6 shows the purified muscle membrane ADP-ribosyltransferase. SDS-PAGE visualized by silver-staining demonstrated that muscle membrane mono-ADP-ribosyltransferase has two isoforms with Mr = 39,000 ± 500 and 38,500 ± 500 designated α and β, respectively as described later (Figures 15 and 16).

**B. Validation of Zymography**

1. **The zymographic in situ gel assay**

The monoADP-ribosyltransferase zymographic assay was originally described (19) as the detection of [P-32]ADP-ribosylated histone that had precipitated in the region of the gel where ADP-ribosyltransferase was localized. Zymography is a method of choice when the presence of other components such as protein, lipid, or metals may interfere with the activity (19). The enzyme, isolated by electrophoresis, is essentially free from stimulation or interference from other components in a complex biological milieu. We characterized the zymographic assay more thoroughly than original report of the assay (19) to assure its accuracy for the detection of monoADP-ribosyltransferase. Parameters studied included linearity, hydroxylamine sensitivity, inhibition by 3-aminobenzamide and
histone stimulation as well as a more thorough definition of the substrate.

Figure 5. Qualitative summary of the purification. Silver stained PAGE of fractions representing the six stages of purification. Identities and amounts of protein loaded were: lane 1, 15 K x g super (20 ug); lane 2, 100 K x g pellet (22 ug), lane 3, 0.3 % doc extract (12 ug); lane 4, DE-52 pool (10 ug); lane 5, concanavalin A eluate (5 ug); lane 6, 3ABA eluate (4 ug); and lane 7, Mr stds.
2. **Linearity**

The zymographic reaction was linear with respect to time for up to 60 min, with a maximal reaction seen at 4 h as studied in the present investigation (Figure 6).

![Figure 6](image)

**Figure 6.** Time course of zymographic reaction. Gel slices, from the 38,000 - 39,000 Da region of lanes in which 11 ug protein (monoADP-ribosyl-transferase, con A eluate) was electrophoresed, incubated for the indicated times, fixed, washed and counted as described in "Methods."
3. **Hydroxylamine sensitivity**

Sensitivity of the linkage between ADP-ribose and the acceptor to neutral hydroxylamine is a criterion for the determination of what type of amino acid was ADP-ribosylated (27). Cysteine specific ADP-ribosylation is relatively insensitive as are the nonenzymatic Schiff base reactions that can occur between the reducing end aldehyde of ADP-ribose generated from hydrolyzed NAD and free amino groups. Arginine specific ADP-ribosylation is determined by a partial (50%) loss of label by incubation with 0.5 M hydroxylamine for 1 h at 37°C. ADP-ribosylated carboxyl groups, as in the poly-ADP-ribosyltransferases are completely labile to 0.5 M hydroxylamine. As shown in Figure 7, there is a time-dependent release of 50% of the label from the hydroxylamine treated auto-ADP-ribosylated protein as compared to the NaCl treated protein. This indicates that the zymographic auto-ADP-ribosylation occurs on arginine residues.

4. **3-aminobenzamide inhibition**

The ADP-ribosyltransferase zymographic reaction was inhibited with 3-aminobenzamide, a known inhibitor of mono-ADP-ribosyltransferase in the millimolar range (29). Figure 8 is a Dixon-Webb plot of the auto-ADP-ribosylation with variable concentrations of 3-aminobenzamide (Ki = 14 mM). There was a nearly linear decrease in the labeling of enzyme
in the gel slices with increasing inhibitor concentration, indicating a specific inhibition of ADP-ribosyltransferase.

Figure 7. Hydroxylamine sensitivity. Zymographically reacted preparative-gel slices containing [P-32]ADP-ribosylated transferase were electroeluted (20 ug) and incubated in 0.5 M NaCl (○) or 0.5 M neutral hydroxylamine (●) as described in "Methods."
Panel A, autoradiogram; Panel B densitometry scans.
Figure 8. 3-aminobenzamide inhibition of zymography.

Gel slices from the 38,000 - 39,000 Da region of lanes to which 11 ug protein (monoADP-ribosyltransferase, con A eluate) was electrophoresed, incubated in the zymographic reaction conditions with the indicated concentrations of 3-aminobenzamide for 4 h, fixed, washed and Cerenkov counted. Incorporation of [P-32] was plotted 1/cpm versus inhibitor concentration in a Dixon-Webb plot as described in "Methods."
5. **Histone stimulation**

The zymographic auto-ADP-ribosylation of membrane mono ADP-ribosyltransferase was found to be stimulated by histone as shown in Figure 9.

![Graph showing histone stimulation.](image)

**Figure 9.** Histone stimulation. Gel Slices from the 38,000 - 39,000 Da region of lanes to which 11 ug protein (monoADP-ribosyltransferase, con A eluate) was electrophoresed, zymographically incubated in the indicated concentrations of histone, fixed, washed and counted as described in "Methods."
6. **Auto-ADP-ribosylation**

The notion of ADP-ribosylated histone precipitating in the region of the gel where the enzyme was present as originally described (19) was tentatively shown to be incorrect by the results presented in Figure 10. Gel slices incubated with the full complement of buffer components, including histone, were carried through the zymographic assay, electroeluted, and re-electrophoresed (Figure 10, lane 1) with histone control in an adjacent lane (Figure 10, lane 2). The Mr = 38,000 - 39,000 band was labeled as were some components at the tracking dye front (Figure 10, lane 3). No radioactive components were found corresponding to the molecular weight forms of the proteins in the histone preparation. The biochemical nature of the low molecular mass component at the tracking dye front was not positively identified but could be residual [P-32] NAD or some ADP-ribose lost from the enzyme during the second electrophoretic step. The results from these findings suggest that the ADP-ribosyltransferase is auto-ADP-ribosylated in the presence of histone.

7. **Confirmation**

The identity of the Mr = 38,500 - 39,000 bands (α + β forms) as mono-ADP-ribosyltransferase and the validity of the zymographic assay was positively confirmed by incubation of renatured enzyme in gel slices from the above Mr range with 2
mM DEA-BAG, 10 mM NAD, and 100 mM phosphate buffer, pH 7.5, for 0, 2 and 4 h. Reverse-phase HPLC analysis performed as described in "Experimental Procedures," confirmed the conversion of DEA-BAG to ADP-ribosylated DEA-BAG with an approximate specific activity about 10% that of the starting material.

Figure 10. Auto-ADP-ribosylation. Zymographic gel slices from the 38,000 -39,000 region of several lanes containing a total of 60 ug protein (ADP-ribosyltransferase, con A eluate) were electroeluted (18 ug) and re-electrophoresed. Lane 1 is the silver stain of the re-electrophoresed sample, lane 2 is the silver stain of 5 µg histone (Sigma, type VIII), and lane 3 is the autoradiogram of lane 1. The arrows denote the location of the 38,500 Da band.
Figure 11 shows the HPLC chromatograms obtained after incubations for A) 0 h; B) 2 h; and C) 4 h. Product peaks eluted at retention times 16 and 17 min, internal standards eluted at 14.25 and 15 min, and unconverted substrate eluted at 20 to 25 min. Incubations carried out on other regions of the gel showed no enzymatic activity.

C. Characterization

1. Sensitivity to reduction

The zymographic assay was quite reproducible with regard to the pretreatment of the cholera toxin and muscle mono-ADP-ribosyltransferase with reducing agent. As shown in Figure 12, cholera toxin displayed a marked enhancement of activity in the presence of β-mercaptoethanol (lanes 2 and 4 with and without β-mercaptoethanol respectively). In contrast, the muscle mono-ADP-ribosyltransferase showed a marked decrease in activity with a β-mercaptoethanol pretreatment (Figure 12, lanes 1 and 3 with and without β-mercaptoethanol respectively). Near overloading of the gel was necessary to detect a signal for the muscle transferase under reducing conditions. Under reducing conditions, the cholera toxin A subunit split into the catalytic A1 subunit Mr = 21,000 and the A2 subunit Mr = 7,000. No shift in Mr was detected for the muscle membrane ADP-ribosyltransferase with reduction, indicating a monomeric catalytic subunit.
Figure 11. DEA-BAG HPLC assay chromatograms of renatured gel slices. The reaction mixture was incubated for (A) 0 h, (B) 2 h, and (C) 4 h as described in "Methods." The anomeric ADP-ribosylated DEA-BAG product peaks had retention times of 16 and 17 min (shown by arrow). Internal standards had retention times of 14.5 and 15 min. Unconverted DEA-BAG had a retention time of 20 - 23 min.
Figure 12. Zymography autoradiogram: β-mercaptoethanol pretreatment of cholera toxin A and membrane muscle ADP-ribosyltransferase. Lanes 1 and 3 were 20 ug each of muscle membrane ADP-ribosyltransferase (con A eluate) with and without pretreatment in sample buffer containing 5% β-mercaptoethanol, respectively. Lanes 2 and 4 were and 5 ug cholera toxin A subunit with and without pretreatment in sample buffer containing 5% β-mercaptoethanol, respectively.
A time dependent loss of ADP-ribosyltransferase activity was monitored by the quantitative DEA-BAG HPLC assay as shown in Figure 13. Transferase was preincubated in 10 mM dithiothreitol for 0 to 6 hours followed by assay in 2 mM DEA-BAG, 10 mM NAD and 100 mM phosphate, pH 7.5 and analysis by reversed phase HPLC as described in "Methods." Inclusion of NAD with the dithiothreitol provided a partial protection of inactivation. The results shown in Figures 12 and 13 indicate the likely presence of a buried intramolecular disulfide that is required for full activity.

2. Zymographic summary of the last stages of the purification

A qualitative summary of the last stages of the purification is shown in Figure 13. Panel A is the silver-stained gel, and panel B is the autoradiogram of the same gel carried through the zymographic in situ mono-ADP-ribosyltransferase assay. Lanes 1 through 3 are the last three stages of purification. Near overloading of the mini-gel with crude fractions (earlier stages) allowed detection of just one ADP-ribosyltransferase band with the zymography when overexposed to the film with intensifying screens (data not shown). These procedures were employed to display the sensitivity and specificity of the zymographic assay used in this investigation. The purified enzyme (lane 3) migrated as
a single silver-stained protein band (panel A) which aligned perfectly with the zymographic signal as seen in panel B. The Mr (avg) = 39,000 ± 500. The purified protein could not be seen after coomassie staining and required silver-staining for visualization.

3. Isoforms with two molecular weights

An apparent doublet of active enzyme in the penultimate step of purification (con A eluate) was localized utilizing the zymographic assay and silver-stained gels. The two bands had Mr = 39,000 ± 500 and 38,500 ± 500 and were designated α and β respectively. The 3-aminobenzamide agarose column resolved the α and β forms almost entirely. As seen in Figure 15, the 3-aminobenzamide fall through active band (lane 1) had a Mr = 38,500 ± 500 (β) whereas the 3-amino-benzamide agarose retained fraction had a Mr = 39,000 ± 500 (α). Both forms could auto-ADP-ribosylate in the zymographic assay.

4. ADP-riboseylhydrolase conversion of α to β

The difference in Mr of 500 could be accounted for if the ADP-ribosyltransferase α was an endogenously auto-ADP-ribosylated form of ADP-ribosyltransferase β because the molecular mass of ADP-ribose is 559. To test this theory, we treated the con A eluate (α + β) with a partially purified ADP-ribosylhydrolase before electrophoresis and zymography.
Figure 13. Inactivation of ADP-ribosyltransferase by reduction. ADP-ribosyltransferase (27,000 U in 100 ul) was preincubated at 30 oC in (■) buffer alone (100 mM phosphate, pH 7.5); (□) 10 mM NAD in buffer; (▲) 10 mM dithiothreitol in buffer; or (△) 10 mM dithiothreitol and 10 mM NAD in buffer. At the indicated times, aliquots were assayed as detailed in "Methods."
Figure 14. Zymographic summary of purification. Panel A, silver stained gel of fractions representing the last three stages of purification; Panel B, autoradiogram of the same gel carried through the zymographic assay as described in "Methods." Lane 1, DE-52 pool (10 µg); lane 2, con A eluant (5 µg); lane 3, 3ABA eluant (4 µg).
Figure 15. Autoradiogram of zymographic analysis of ADP-ribosyltransferase α and β. Lane 1 was the 3-aminobenzamide agarose fall through (β, 10 µg) and lane 2 the eluant (α, 4 µg), carried through the zymography as described in "Methods."

The results are shown with an autoradiogram of the zymographic assay in Figure 16. The average molecular mass of ADP-ribosyltransferase α + β decreased by about 500 daltons to a mass similar to that assigned as ADP-ribosyltransferase β when incubated with ADP-ribosylhydrolase (Figure 16, lane 2).

In addition to a shift in molecular mass, the ADP-ribosyltransferase β displayed a greater activity than the mixture of α and β as determined by scintillation counting.
Figure 16. Conversion of isoform α to β: ADP-ribosylhydrolase incubation with ADP-ribosyltransferase; lane 1 was ADP-ribosyltransferase (con A eluate, 12 ug) incubated alone, lane 2 was ADP-ribosyltransferase (con A eluate, 12 ug) incubated with ADP-ribosylhydrolase (5 ug, 493 U/mg) lane 3 was ADP-ribosylhydrolase incubated alone before electrophoresis and zymography as described in "Methods." Scintillation counting of the gel slices were 236 cpm (lane 1), 1100 cpm (lane 2), and 130 cpm (lane 3). ADP-ribosyltransferase α and β had Mr = 39,000 and 38,500, respectively.
5. **ADP-ribosyltransferase is a glycoprotein**

ADP-ribosyltransferase (10 ug), electrophoretically purified from 3-aminobenzamide fall through, was sent to Dr. Kevin Campbell's lab (Dept. of Physiology and Biophysics, Iowa City) for analysis. The sample was electrophoresed in a lane adjacent to dihydropyridine receptor, pure calsequestrin and triads, blotted to nitrocellulose and probed with horse radish peroxidase conjugated to concanavalin A. As shown in Figure 17, transferase (mADPRT) was a positive signal as were the dihydropyridine receptor and triads. Pure calsequestrin, which is not reactive with concanavalin A, did not react.

![Figure 17. Concanavalin A recognition of glycoprotein.](image)
IV. DISCUSSION

An integral membrane-associated arginine specific mono-ADP-riboseyltransferase has been purified to ≥ 95% homogeneity. We have determined some of its catalytic and structural properties making this the first report of the purification of the mammalian form of the enzyme. The specific activities of the purified α form, 1.9 umol/min·mg, and β-form, 6.0 umol/min·mg, are about 10 - 30 X higher than that for the mono-ADP-ribo­

syltransferase purified from hen liver nuclei, 0.197 umol/min·mg (36); about the same as the cys-specific transferase purified from human erythrocyte, 8 umol/min·mg (4) and about 50 X less than that from turkey erythrocyte, 353 umol/min·mg (5). The exact assays used to determine the enzymatic activities in the various studies differ and care should be taken in making close comparisons.

Kinetic parameters were determined from secondary plots of double reciprocal plots (16). The Km values for the muscle monoADP-ribosyltransferase were found to be 1.2 mM for LAME and 0.56 mM for NAD. The Vmax was 40 umol/min/mg. The Km for NAD was about 80 times higher than that for ADP-ribo­

syltransferase from turkey erythrocyte (9), 8 times higher than transferase from hen liver nuclei (36) and 8.6 times higher than the cys-specific transferase from human erythrocyte. The Vmax for the turkey erythrocyte mono-ADP-ribo­

syltransferase, using agmatine as the co-substrate, was 31 umol/min/mg (9).
The Vmax for the cys-specific transferase was 34.5 umol/min/mg using cysteine methyl ester as the acceptor (4).

The 645-fold purification relative to the microsomal fraction suggests a moderate abundance of the enzyme in the muscle microsome. This could reflect a physiological role in the regulation of Ca++ transport (36) in support of muscle contraction processes. The muscle enzyme is more abundant than the turkey erythrocyte ADP-ribosyltransferase which required a 544,000 fold purification (5) and the cys-specific transferase from human erythrocyte which required a 35,000 fold purification (4). The mono-ADP-ribosyltransferase purified from hen liver nuclei (34) required a 640 fold purification relative to isolated nuclei, which suggests a similar abundance as the muscle ADP-ribosyltransferase. The low concentration of monoADP-ribosyltransferase in the erythrocyte may be due in part to the presence of hemoglobin, which constitutes 90% of the solid mass of the erythrocyte (35). Until the physiological role is assigned to the various classes of endogenous ADP-ribosyltransferases, there is no assurance that the mammalian ADP-ribosyltransferase will resemble that from avian sources with respect to relative abundance, specific activity and regulation.

The Mr = 38,500 - 39,000 ± 500 of the isozymes isolated in these studies was higher than the mono-ADP-ribosyltransferases isolated from erythrocyte Mr = 28,000 - 32,000 (1,4,5),
identified in *Xenopus* skeletal and heart muscle Mr = 32,000 (18), or cholera toxin A1 subunit Mr = 21,000 (1). The enzyme is glycosylated as determined by the complete binding of the horse radish peroxidase conjugated concanavalin A to transferase bound to nitrocellulose. It also displayed an inability to be visualized by Coomassie staining and required silver-staining. The differences of Mr between the erythrocyte and skeletal muscle membrane ADP-ribosyltransferase may be a function of the degree of glycosylation or the presence of a signal peptide dependent upon the biosynthetic route and the post-translational processing the enzyme undergoes prior to insertion into the target membrane (33). The muscle microsomal membrane preparation used in this investigation was prepared with 100,000 x g centrifugal force for pelleting of the homogenate after a low-speed centrifugation and filtration. These forces pellet membrane components from the plasma membrane, sarcoplasmic reticulum, and sarcolemma (23). The two ADP-ribosyltransferase isoforms reported in this study could be from different subcellular locations.

Closer inspection of the differences between the 3-aminobenzamide agarose fractions revealed a subtle nature of the enzyme. Close to 80% of the activity did not bind to the 3-aminobenzamide agarose column. The remainder of the activity was bound to and eluted from the column with either 25 mM 3-methoxybenzamide or 2 M KCl in the regeneration step.
Zymography of silver-stained gels revealed doublet bands at Mr = 39,000 and 38,500 ± 500. The 3-aminobenzamide agarose column resolved these two molecular weight forms and they were termed mono-ADP-ribosyltransferases α and β for the higher and lower Mr forms, respectively.

Attempts to bind the β form to the 3-aminobenzamide column were unsuccessful, both before and after auto-ADPribosylation in solution. Although mono-ADP-ribosyltransferase was found to auto-ADP-ribosylate in the zymographic assay and in solution, the stoichiometry is thought to be > 1. Further studies on the conformation of ADP-ribosyltransferase α and β would help define the preferential binding of the α form to the 3-aminobenzamide column. It could be that some specific factor such as ARF (31) or another membrane protein (32) is required for the auto-ADP-ribosylation to occur to produce the appropriate conformation. Alternatively, the auto-ADP-ribosylation site in the α form may block the binding of some other protein that restricts binding to the 3ABA column. A 58,000 Da protein, as determined by SDS-PAGE, was found to always co-elute with the β form in the 3ABA fall through fractions. This protein was positively identified as calsequestrin by N-terminal sequencing and western blotting (data not shown). If the auto-ADP-ribosylation site in the α form blocks the interaction with the 58,000 Da protein, which blocks binding to the 3ABA column, then this could explain
the binding of the α (auto-ADP-ribosylated) form of the transferase to the column.

The turkey erythrocyte mono-ADP-ribosyltransferase is stimulated by histone (30) and this property is shared by the membrane muscle mono-ADP-ribosyltransferase auto-ADP-ribosylation. The relative dependence of isozymes α and β upon histone stimulation of activity towards exogenous substrates is under further investigation. The muscle membrane ADP-ribosyltransferase was found to be inactivated by reducing agents. This was found to be in contrast to cholera toxin A subunit, which requires reduction for release of the free catalytic subunit. With reduction, the muscle ADP-ribosyltransferase demonstrated a marked drop in activity yet underwent no shift in Mr. This indicates that the catalytic subunit is a monomer, as shown with the other transferases (1,5,18,34) but must contain either an intramolecular disulfide or disulfide linkage with a very small molecule such as glutathione for full activity. The inactivation kinetics were very slow (T 1/2 > 1 Hr, Figure 13), indicating a relatively inaccessible site of reduction. This property of the membrane ADP-ribosyltransferase may be an indication of regulation by either the oxidation state or degree of S-thiolation of the enzyme.

It seems likely that many of the arginine specific transferases will demonstrate different substrate
specificities and biological functions. ADP-ribosylation of transducin by the arginine specific transferase purified from turkey erythrocyte was shown to inhibit the GTPase activity of the G-protein (34). This study indicated that the endogenous ADP-ribosyltransferases may play a critical role in the regulation of G-protein mediated signal transduction. The specificity of the erythrocyte transferase towards transducin was found to be different from that of cholera toxin which does not inhibit the GTP binding activity of transducin as does the erythrocyte transferase (34). The metabolic role of the muscle membrane associated mono-ADP-ribosyltransferase has not been clearly defined. One study (35) reported an NAD-dependent suppression of Ca++-dependent ATPase in preparations containing both the ATPase and the ADP-ribosyltransferase which were partially purified from skeletal muscle sarcoplasmic reticulum and reconstituted into vesicles. Since Ca++ transport plays a central role in muscle contraction, such regulation of the Ca++ ATPase by ADP-ribosyltransferase may reflect a adrenergic or muscarinic signal transduction effector system.

It is very likely that the membrane mono-ADP-ribosyltransferase serves some metabolic or signal transductional role since the ADP-ribosylhydrolase activity is also found in skeletal muscle. Strict regulation and substrate specificity of the enzyme is suspected and warranted because of the
resemblance of this enzyme to certain pathological toxins. This purified enzyme will provide a tool for further investigation and understanding of the role of endogenous intramembrane mono-ADP-ribosyltransferase in signal transductional events.
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