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NADP IMPROVES THE EFFICIENCY OF CHOLERA TOXIN CATALYZED ADP-RIBOSYLATION IN LIVER AND HEART MEMBRANES

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Guanine nucleotide binding proteins (G-proteins) can be identified by their ability to be ADP-ribosylated using [32P]NAD as the substrate and bacterial toxins as catalysts. This labelling, when performed in liver and sarcolemma membrane preparations, can be complicated by competing enzymes which degrade NAD, making it unavailable to participate in the desired reaction. The addition of NADP in reaction mixtures markedly slows the degradation of NAD, but does not compete with NAD in cholera toxin labelling of stimulatory G-protein. The efficiency of cholera toxin labelling is improved to the extent that saturation curves may be constructed, allowing the quantitation of ADP-ribosylation sites in membranes.

The use of bacterial toxins in identifying functional and structural properties of GTP-binding proteins (G-proteins) has proven invaluable over the past decade. For example, the alpha subunits of the stimulatory (G_s-alpha) and inhibitory (G_i-alpha) proteins associated with membrane bound adenylate cyclase can be ADP-ribosylated by cholera toxin and pertussis toxin respectively. These reactions require the presence of NAD, GTP or certain of its analogues, the toxin, and in the case of G_s-alpha, an ADP-ribosylation factor (1,2). When [32P]NAD is used as a substrate, these alpha subunits can be identified using sodium dodecyl sulfate polyacrylamide gel electrophoresis and autoradiography (3,4).

Radiolabelling has been performed on membrane preparations from many tissues, as well as solubilized and purified preparations of these proteins. A particular problem arises when any of the substrates involved in the transfer of ADP-ribose to the acceptor are consumed. As noted by Fleming and Watanabe (1), purified canine cardiac sarcolemma has levels of NAD degradative activity that are 500-fold greater than erythrocyte membranes. This necessitates dilution of membrane protein to concentrations less than

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20ug/ml to demonstrate labelling of G_s-alpha with cholera toxin. Even at these dilutions, significant degradation of NAD occurs thus preventing quantitative intoxication or radiolabelling of G_s-alpha. In this report we assess the activity of enzymes which cleave NAD in several types of purified plasma membranes. Further, we show that addition of NADP to reaction mixtures inhibits degradation of NAD, but that does not interfere with cholera toxin-mediated ADP-ribosylation of G_s-alpha. This results in markedly improved efficiency of radiolabelling of G_s-alpha such that quantitation of ADP-ribosylation sites is possible.

METHODS

1. Materials

[32P]NAD was obtained from New England Nuclear. All other reagents were obtained from Sigma.

2. Membranes

S49 mouse lymphoma cells and a variant, cyc-, were obtained from the San Francisco Cell Repository and membranes were prepared as described (5). Adult rat liver membranes were made from crude homogenates of freshly excised liver and purified on Percoll gradients as described (6). Cardiac sarcolemma was prepared from adult mongrel dogs after the method of Jones et al (7). Protein determinations were made using the method of Lowry (8).

3. Chromatography

Reaction mixtures were checked for the presence of [32P]NAD by chromatography with polyethylenimine-cellulose thin layer plates in a solvent of 0.40M lithium chloride.

4. Cholera toxin [32P]NAD Labelling of Membranes

Radiolabelling of membranes was performed in the presence or absence of cholera toxin by a modification of the method of Rebois (9). The final assay mixture (250 ul) contains KPi 50 mM, Hepes 10 mM (pH 7.4), NaCl 27 mM, cholera toxin 50-200 ug/ml (as indicated), GTP 100 uM, thymidine 5mM, +/- NADP 500 uM and in the case of sarcolemma, an ATP/GTP regenerating system consisting of ATP 2mM, phoshocreatine 10 mM and creatine phosphokinase 10 ug/ml. No difference in labelling was noted when 5'-guanylylimidodiphosphate was substituted for GTP. Labelling of sarcolemma requires the addition of an ADP-ribosylation factor (1). This was provided by the addition of S49 cyc- membranes (which are genetically deficient in G_s-alpha) in a membrane protein ratio of 4 to 1 over sarcolemma. Reactions were begun by the addition of [32P]NAD to a final concentration of 5uM (50 uCi/ml) and carried out for the indicated times. Reactions were terminated by the addition of ice cold buffer (Hepes 20 mM/$MgCl_2$ 2mM/ EDTA 1mM) with 1 mM NAD, centrifuged at 20,000 x g for 20 minutes and then resuspended in 50 ul of sample buffer. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis was performed according to Laemmli (9).

RESULTS

Figure 1 demonstrates the loss of [32P]NAD label as shown on PEI-cellulose TLC. Liver and cardiac membranes rapidly degrade NAD as seen

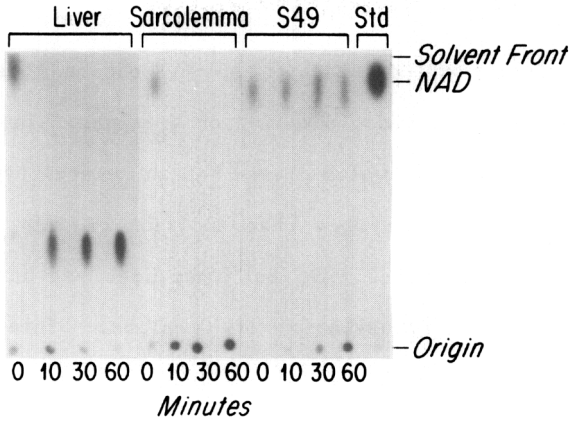


Figure 1

Polyethylenimine cellulose thin layer chromatography of reaction mixtures. Reaction mixtures (0.5 ul) were spotted at the origin of thin layer plates and run in a solvent of 0.4 M $LiCl_2$ for 10 cm. Plates were dried and exposed to film for 30-60 minutes. The panels show degradation of 32P-NAD over the times indicated when membrane protein concentrations were 0.5 mg/ml for S49, liver, and cardiac (sarcolemma) membranes. An NAD standard with 5-fold more counts is also included.

by the loss of label at the position marked by the NAD standard to one much nearer or at the origin, when membrane concentrations are 0.5 mg/ml. This loss occurs rapidly within the first 5-15 minutes of incubation. In contrast, S49 membranes do not degrade NAD as rapidly, and significant amounts of this substrate remain after one hour of incubation, when the same membrane protein concentration is used.

In Figure 2, the effect of membrane dilution is seen. When liver membranes are diluted by a factor of 2.5, to 0.2mg/ml, NAD is still rapidly consumed in reaction mixtures. However, when sarcolemma or liver is diluted 50-fold to 0.04 mg/ml, degradation of NAD is reduced such that appreciable amounts remain even after one hour of incubation (data for liver not shown). Even with these reduced protein concentrations, however, NAD is still degraded as evidenced by appearance of label at lower R_f values. The presence or absence of cholera toxin in concentrations ranging from 50ug/ml to 200ug/ml does not effect the degradation of NAD (data not shown). When NADP is included in reaction mixtures, the degradation of NAD is slowed markedly or entirely prevented (Figure 2). In liver membranes at 0.2 mg/ml,

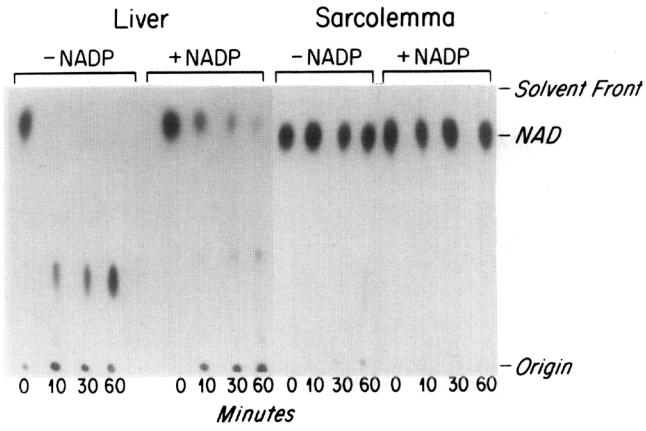


Figure 2

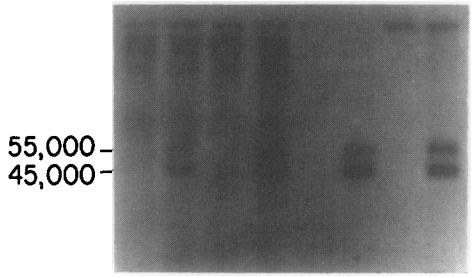
Liver and sarcolemma reaction mixtures run on polyethylenimine cellulose TLC. Protein concentrations were 0.2 mg/ml for liver and 0.04 mg/ml for sarcolemma. Reactions were run in the absence and presence of NADP as indicated and spotted at the indicated times. Plates were run, dried, and exposed to film as described in Methods and in Figure 1.

[32P]NAD remains in significant amounts after one hour when NADP is present at 0.5 mM as compared to its complete disappearance within 15 minutes when NADP is absent. This effect is also seen in sarcolemma. The degradation of [32P]NAD, apparent even at low membrane protein concentrations, is almost completely inhibited when NADP is included in the assay mixture. In contrast, isobutylmethylxanthine (IBMX) at 1 mM had no effect in inhibiting the degradation of NAD (data not shown).

It is important to show that NADP does not compete with NAD as a substrate in the cholera toxin-mediated transfer of ADP-ribose to G_s-alpha. This is seen in Figure 3. In S49 membranes, the presence of NADP does not effect the extent of the labelling of G_s-alpha, which appears as two species of 45 and 55 kilodaltons. Further, in liver membranes, the extent of labelling of these two species is increased almost two-fold in the presence of NADP, presumably because the cholera toxin-catalyzed reaction can occur more completely when [32P]NAD remains available for longer periods of time.

Under appropriate conditions, labelling of the cholera toxin substrate can be saturated. Figure 4 demonstrates the time course of cholera toxin specific incorporation of [32P]ADP-ribose in cardiac sarcolemma. Under the

Membrane	S49				Liver			
Cholera Toxin	−	+	−	+	−	+	−	+
NADP	−	−	+	+	−	−	+	+
G_s Alpha Density		1.00		1.02		1.00		1.92



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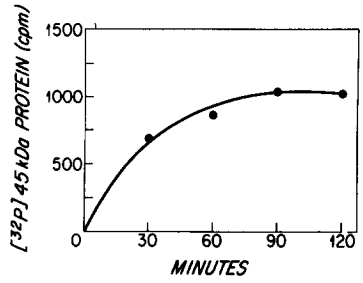

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Figure 3

Sodium dodecyl sulfate gel electrophoresis of reaction mixtures which were prepared as described in Methods with and without cholera toxin (50 ug/ml) and with and without NADP (0.5 mM) as shown using S49 and liver membranes (10 ug per lane.) Gels were dried and exposed to film for 12 hours and the resulting autoradiogram was scanned. The portions of the autoradiogram containing the bands specifically labelled by cholera toxin were scanned and the relative densities are listed. For each membrane type the density of the cholera toxin-specific bands in the assay without NADP was set equal to 1.0.

Figure 4

Incorporation of cholera toxin specific counts into sarcolemma membranes. Sarcolemma (0.04 mg/ml membrane protein, 10 ug total) was incubated as described in Methods with and without cholera toxin. The reactions were terminated at the times shown and run on 13% sodium dodecyl sulfate polyacrylamide gels. The 45 kilodalton regions of all lanes were excised and counted using liquid scintillation counting. Counts from lanes from controls without cholera toxin at a given time were subtracted from those with cholera toxin to yield cholera toxin-specific counts.

conditions of the assay, which has relatively large amounts of cholera toxin (200 ug/ml) and a moderate concentration of NAD (5uM-50uCi/ml), labelling of the 45 kDA species is complete after 90 minutes, under conditions where there is no detectable loss of [32P]NAD.

CONCLUSION

The cholera toxin-catalyzed incorporation of ADP-ribose into G_s-alpha in purified liver and sarcolemma membranes is complicated by enzymatic cleavage of the substrate, NAD. Carrying out these reactions at dilute protein concentrations only partially alleviates this problem. The data we present here show that inclusion of NADP in reaction mixtures markedly reduces

degradation of NAD, and yet does not compete with it as a substrate in the ADP-ribosylation of G_s by cholera toxin. This is expected because cholera toxin cannot use NADP as a substrate at the concentrations used in our assays (10). When [32P]NAD is used in the reaction for radiolabelling, the extent of labelling in liver membranes is substantially increased owing to the greater availability of substrate. Further, under conditions where a GTP/ATP regenerating system is included in the assay it is possible to saturate the labelling of G_s-alpha in cardiac sarcolemma if NADP is present and the membranes are sufficiently dilute. This allows quantitation of ADP-ribose acceptor sites in membranes which have significant NAD degradative activity.

The identity of the enzymes which degrade NAD is not clear. However, since IBMX does not inhibit NAD cleavage in our assays, it is likely that degradation is primarily due to NAD glycohydrolase rather than phosphodiesterase activity.

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