Inactivation of Glutamine Synthetases by an NAD:Arginine ADP-Ribosyltransferase*

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Glutamine synthetase from ovine brain has a critical arginine residue at the catalytic site (Powers, S. G., and Riordan, J. F. (1975) Proc. Natl. Acad. Sci. U. S. A. 72, 2816–2820). This enzyme is now shown to be a substrate for a purified NAD:arginine ADP-ribosyltransferase from turkey erythrocyte cytosol that catalyzes the transfer of ADP-ribose from NAD to arginine and purified proteins. The transferase catalyzed the inactivation of the synthetase in an NAD-dependent reaction; ADP-ribose and nicotinamide did not substitute for NAD. Agmatine, an alternate ADP-ribose acceptor in the transferase-catalyzed reaction, prevented inactivation of glutamine synthetase. MgATP, a substrate for the synthetase which was previously shown to protect that enzyme from chemical inactivation, also decreased the rate of inactivation in the presence of NAD and ADP-ribosyltransferase. Using [32P]NAD, it was observed that approximately 90% inactivation occurred following the transfer of 0.89 mol of [32P]ADP-ribose/mol of synthetase. The erythrocyte transferase also catalyzed the NAD-dependent inactivation of glutamine synthetase purified from chicken heart; 0.60 mol of ADP-ribose was transferred per mol of enzyme, resulting in a 95% inactivation. As noted with the ovine brain enzyme, agmatine and MgATP protected the chicken synthetase from inactivation and decreased the extent of [32P]ADP-riboseylation of the synthetase.

These observations are consistent with the conclusion that the NAD:arginine ADP-ribosyltransferase modifies specifically an arginine residue involved in the catalytic site of glutamine synthetase. Although the transferase can use numerous proteins as ADP-ribose acceptors, some characteristics of this particular arginine, perhaps the same characteristics that are involved in its function in the catalytic site, make it a favored ADP-ribose acceptor site for the transferase.

The catalytic activity of enzymes located at critical points in metabolic pathways is, in many cases, regulated by covalent modification. Certain bacterial toxins perturb cellular metabolism by catalyzing the transfer of ADP-ribose from NAD to a key enzyme, thereby altering its activity (1–4). Cholera and Escherichia coli heat-labile enterotoxin catalyze the ADP-ribosylation of a regulatory component of the hormone-sensitive adenylate cyclase system (1, 5–7); evidence from studies with model substrates is consistent with the hypothesis that the target amino acid is an arginine (8, 9). Diphtheria toxin and Pseudomonas exotoxin A inhibit protein synthesis in susceptible animal cells by ADP-ribosylating elongation factor II (2, 3); in this case, the target amino acid is a modified histidine residue (10).

Animal cells contain endogenous enzymes that catalyze the mono-ADP-ribosylation of proteins in a reaction analogous to that catalyzed by the bacterial toxins (11–13). The role of the mono-ADP-ribosyltransferases is not clear. Two transferases that have been purified to homogeneity from turkey erythrocytes catalyze the ADP-ribosylation of proteins, arginine, and low molecular weight guanidino compounds; amino acids, such as lysine and histidine, are inactive as ADP-ribose acceptors (11, 14).

A number of enzymes have critical arginine residues that participate in catalysis. These have been identified by the use of chemical reagents that specifically react with arginine (15). We used glutamine synthetase, which contains an active site arginine that is reactive with such reagents (15), to determine whether this arginine might also be preferentially modified by an ADP-ribosyltransferase.

EXPERIMENTAL PROCEDURES

Materials

Ovine brain glutamine synthetase, ADP-ribose, NAD, NADP, ATP, L-glutamate, and phenylmethylsulfonyl fluoride were purchased from Sigma; Tricine from Calbiochem-Behring; nicotinamide from Nutritional Biochemical Corp.; frozen chicken hearts from Pel-Freez; MgCl2 and NH4Cl from Fisher; phenyl-Sepharose from Pharmacia; DE52 from Whatman; Uitrolg AcA 34 from LKB; ATP-genes from P-L Biochemicals; [carbonyl-14C]NAD (53 mCi/mmol), [adenine-U-14C]NAD (287 mCi/mmol), and L-[U-14C]glutamic acid (280 mCi/mmol) from Amersham Corp.; and [32P]NAD (24 Ci/mmol) from New England Nuclear.

Methods

Assays—Erythrocyte ADP-ribosyltransferase was assayed in a total volume of 0.3 ml containing 50 mM potassium phosphate (pH 7.0), 2 mM agmatine, 32.4 μM [carbonyl-14C]NAD (~40,000 cpm), ovanbumin (1 mg/ml), and 300 mM NaCl (16). The reaction was initiated with ADP-ribosyltransferase (~1 ng). After incubation at 30°C for 30 min, two 0.1-ml samples were transferred to columns to isolate [carbonyl-14C]nicotinamide (17).

Glutamine synthetase was assayed by a modification of the method of Tiemeier and Milman (18). Assays (total volume 0.2 ml) contained 50 mM Tricine (pH 7.6), 20 mM sodium glutamate, L-[U-14C]glutamic acid (150,000 cpm), 20 mM MgCl2, 15 mM ATP, 4 mM NH4Cl, and ~2 μg of glutamine synthetase. After 30 min at 30°C, 0.1 ml of H2O was added and a 0.25-ml sample was transferred to a column (0.5 × 4 cm) of AG 1-X2. The [14C]glutamate was eluted with two 1.2-ml portions of H2O.

1 The abbreviations used are: Tricine, N-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine; EGTA, ethylene glycol bis(β-aminoethyl ether)-N,N,N′,N′-tetraacetic acid.

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Protein was determined by a modification of the method of Lowry et al. (19) using bovine serum albumin as a standard.

Purification of the NAD:Arginine ADP-Ribosyltransferase—The NAD:arginine ADP-ribose transferase purified from turkey erythrocytes by sequential chromatography on phenyl-Sepharose, carboxymethylcellulose, NAD-agarose, and concanavalin A-agarose as described (12) exhibited one major protein band by sodium dodecyl sulfate-polyacrylamide gel electrophoresis; 1 unit of transferase activity equals 1 μmol of ADP-ribose transferred from NAD to arginine/min at 30 °C.

**TABLE I**

<table>
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<th>Purification step</th>
<th>Protein</th>
<th>Total units</th>
<th>Specific activity</th>
<th>Purification</th>
<th>Yield</th>
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<td>122</td>
<td>8.65</td>
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<td>33</td>
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</tbody>
</table>

**FIG. 1.** Sodium dodecyl sulfate-gel electrophoresis of purified chicken heart glutamine synthetase. Left, glutamine synthetase was subjected to electrophoresis on a 10% polyacrylamide gel in the presence of 0.1% sodium dodecyl sulfate. Right, the log of the molecular weight of standard protein (9) was plotted as a function of Rf to obtain an approximate molecular weight of 41,000 for glutamine synthetase (O). Standard proteins were phosphorylase b (94,000), albumin (67,000), ovalbumin (43,000), and carbonic anhydrase (30,000). Arrow specifies dye front.

**TABLE II**

NAD-dependent inactivation of ovine brain glutamine synthetase by ADP-ribose transferase

Samples of ovine brain glutamine synthetase (1.34 μg) were incubated in a total volume of 0.1 ml containing 5 mM potassium phosphate (pH 7.0) and 5% propylene glycol with or without 1.3 milliunits of transferase and other additions as indicated (MgCl2 (20 mM), ATP (15 mM)). The reaction was initiated by the addition of 0.1 ml of mixture to bring the reactants to the concentrations shown under “Methods;” except for MgCl2 and ATP, all other additions shown in the table were present at 1/2 the given concentration in the assay. All assays were run in quadruplicate.

**TABLE III**

Effect of MgATP and agmatine on inactivation of ovine brain glutamine synthetase in the presence of NAD by ADP-ribose transferase

Samples of ovine brain glutamine synthetase (1.34 μg) were incubated for 5 h at 30 °C in 0.1 ml containing 5 mM potassium phosphate (pH 7.0) and 5% propylene glycol with or without 1.3 milliunits of transferase and other additions as indicated (MgCl2 (20 mM), ATP (15 mM)). The reaction was initiated by the addition of 0.1 ml of mixture to bring the reactants to the concentrations shown under “Methods;” except for MgCl2 and ATP, all other additions shown in the table were present at 1/2 the given concentration in the assay. All assays were run in quadruplicate.

**RESULTS**

Ovine brain glutamine synthetase was inactivated by incubation with NAD and NAD:arginine ADP-ribose transferase (Table II). ADP-ribose and nicotine, products of the enzyme-catalyzed hydrolysis of NAD by the transferase, were inactive (Table II). NADP, which is utilized much less efficiently by the transferase, was not an effective substitute for NAD (Table II). Agmatine, an alternate ADP-ribose acceptor in the transferase-catalyzed reaction, prevented the inactivation of glutamine synthetase (Table III). Addition of McATP, previously shown to block chemical inactivation of synthetase by arginine-specific reagents (15), reduced the rate 1/2 of inactivation of synthetase by transferase and NAD (Fig. 2 and...
The transferase-catalyzed inactivation of glutamine synthetase was associated with the transfer of $^{[32P]}$ADP-ribose from $^{[32P]}$NAD to the enzyme. Maximal inhibition of glutamine synthetase activity of 90% resulted after incubation of this enzyme with transference for ~1 h, was associated with transfer of ~0.89 ± 0.07 mol of ADP-ribose/mol of glutamine synthetase. The ratio of moles of $^{[32P]}$ADP-ribose/mol of glutamine synthetase to percentage inactivation was ~0.99. Glutamine synthetase purified from chicken heart was also inactivated by the transference in an NAD-dependent reaction (Fig. 3). ADP-ribose and nicotinamide could not replace NAD (Table IV). Inhibition of the chicken heart enzyme by transference was maximal by 1 h (Fig. 4) and was dependent on that amount of transference present (Fig. 5). As noted with the ovine enzyme, both agmatine and MgATP protected the synthetase from inactivation (Table IV) and also decreased the extent of ADP-ribosylation (data not shown). In the presence of $^{[32P]}$NAD, 0.60 ± 0.03 mol of ADP-ribose was transferred per mol of glutamine synthetase, resulting in a 95% inactivation of the enzyme. The ratio of moles of $^{[32P]}$ADP-ribose/mol of glutamine synthetase to percentage inactivation was 0.63.

**DISCUSSION**

It was shown by Powers and Riordan (15) that the ovine brain glutamine synthetase has a critical arginine residue based on its inactivation by arginine-specific reagents; in these studies, loss of enzymatic activity was associated with the modification of 3 out of a possible 25 arginine residues. By performing the investigations in the presence of MgATP, a substrate for the synthetase that protected ~1.4 of the arginine residues from modification, it was concluded that a critical arginine residue was present at the active site. In the present study, using the arginine-specific NAD:arginine ribosyltransferase, it was observed that ADP-ribosylation by MgATP was transferred per mol of glutamine synthetase from ovine brain and chicken heart resulted in a loss of enzymatic activity. Both glutamine synthetases were protected from enzymatic inactivation by the addition of a synthetase substrate, MgATP. Saturating concentrations of agmatine, an alternative ADP-ribose acceptor for the erythrocyte transference (14), also blocked inactivation of the synthetases. Specificity of the agmatine effect was verified by demonstrating the formation of ADP-ribose-agmatine, rather than ADP-ribose-glutamine synthetase (data not shown), consistent with the hypothesis that agmatine and glutamine synthetase compete for the active site on the transference. With glutamine synthetases from both sources, it appeared that inactivation resulted from the transfer of ~1 mol of ADP-ribose to 1 mol of enzyme; the reaction thus appeared to be specific.

The NAD:arginine ADP-ribose transferase may be used as a reagent to catalyze the covalent modification in both pure protein and tissue homogenate of arginine residues. The ADP-ribose(arginine) protein bond is relatively stable in acid and at physiological pH (22). In tissue homogenates, enzyme(s) responsible for degradation of the ADP-ribo(arginine) protein bond, if they exist, are relatively inactive under standard assay conditions, and thus unlikely to present a threat to the stability of the ribo(arginine) protein linkage. Reversal of transferase-like reactions requires low pH (5.5–6.0) and high concentrations of nicotinamide (5); it is thus unlikely to proceed under physiological conditions. Since phosphodiesterases that catalyze the degradation of the ADP moiety are common (23), to tag arginine residues in crude extracts it would be preferable to use NAD labeled in the nicotinamide ribose; phosphodiesterase and phosphatase action on ADP-ribose(arginine) protein would result in the formation of ribo(arginine) protein as a radiolabeled end product.

Indirect evidence that ADP-ribosylation might be involved in the regulation of glutamine synthetase in cells was obtained by assessing the effects of lowering cellular NAD levels on glutamine synthetase activity in Chinese hamster ovary cells. Nicotinamide omission from the growth medium produced a 78 and 109% increase in glutamine synthetase activity in two separate experiments coincident with a lowering of the cellular NAD levels by 90%. Nicotinamide deprivation had no effect on cell growth rate over the 12-h treatment period. NAD levels were also reduced by 75% by exposing Chinese hamster ovary cells to 2 mM 6-aminonicotinamide for 24 h. With cells in stationary phase, the compound increased glutamine synthetase levels by 99 ± 15% compared to untreated controls.

In prior studies, it was shown that ADP-ribosylation was affected by nucleotides such as GTP or ATP which, depending on the protein, either increased, decreased, or had no effect on the rate of modification (24). These experiments did not demonstrate an effect of ADP-ribosylation on function. In the present report, it is clear that MgATP blocks the inactivation of glutamine synthetase. Although it was uncertain from the previous studies whether the modification of the proteins had any selectivity other than the presence of a "readily accessible" arginine, the present investigation demonstrates that the transference-catalyzed reaction can be specific for certain arginine residues. Of the 25 arginine residues in ovine brain glutamine synthetase, the erythrocyte transference and phenylglyoxal selectively modified that residue critical for enzymatic activity. It is clear from model studies on the transference-catalyzed ADP-ribosylation of arginine and other low molecular weight guanidino compounds that the environment of the guanidino is a critical determinant of its ability to serve as an ADP-ribose acceptor (14). The presence of negatively charged residues in the vicinity of the guanidino moiety decreased its reactivity in the transference-catalyzed reaction; agmatine and arginine methyl ester were more ef-

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![Fig. 2. Effect of MgATP on the rate of inactivation of ovine brain glutamine synthetase.](http://www.jbc.org/)
synthetase serve as determinants of the pK of the guanidino group and thus its ability to displace nicotinamide from effective substrates than were arginine or guanidinopropionate (14). In addition, the reactivity of an arginine in a protein is in part determined by the nucleophilicity of the guanidino group and thus its ability to displace nicotinamide from NAD⁺. The secondary and tertiary structures of glutamine synthetase serve as determinants of the pK of the guanidino moiety. A decrease in pK would enhance the reactivity of the guanidino group with phenylglyoxal and at the catalytic site of the transferase. In the case of the transferase-catalyzed reaction as opposed to chemical modification, however, the picture is complicated by the fact that the substrate for the transferase is another protein; in order for the critical arginine to be modified, the catalytic site of glutamine synthetase must be accessible to the active site of the transferase. It is thus appealing to speculate that the specificity reflects an in vivo significance for this reaction and a function for the NAD:arginine ADP-ribosyltransferase.
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Inactivation of glutamine synthetases by an NAD:arginine
ADP-ribosyltransferase.

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