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 enzyme from inactivation and decreased the extent of [32P]ADP-ribosylation 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.

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EXPERIMENTAL PROCEDURES

Materials
Ovine brain glutamine synthetase, ADP-ribose, NAD, NADP, ATP, L-glutamic acid, and phenylmethylsulfonyl fluoride were purchased from Sigma; Tricine1 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; Ultrogel AcA 34 from LKB; ATP-agarose from P-L Biochemicals; [carbonyl-14C]NAD (53 Ci/mmol), [adenine-1-14C]NAD (287 Ci/mmol), and L-[U-14C]glutamic acid (280 Ci/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), ovalbumin (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-aminoethylether)-N,N',N'-tetraacetic acid.

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Effect of MgATP and agmatine on inactivation of ovine brain glutamine synthetase by ADP-ribosyltransferase

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.

### TABLE I

| Purification step         | Protein | Total units | Specific activity | Purification yield | Yield % |
|---------------------------|---------|-------------|------------------|--------------------|---------|
| Supernatant               | 5790    | 369         | 0.0657           | 1                   | 100     |
| Phenyl-Sepharose          | 650     | 345         | 0.531            | 8.34               | 94      |
| DE52                     | 191     | 204         | 1.07             | 16.8               | 55      |
| Ultrogel AcA 34          | 79.5    | 192         | 2.42             | 38.0               | 52      |
| ATP-agarose               | 14.1    | 122         | 8.65             | 135.8              | 33      |

### 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 (8) was plotted as a function of Rf to obtain an approximate molecular weight of 41,000 for glutamine synthetase (C). 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-ribosyltransferase

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 for 24 h at 30 °C with or without 1.3 milliunits of transferase and other additions as indicated. Glutamine synthetase activity was then determined as described under "Methods" after addition of assay components in 0.1 ml. Final concentrations of reagents are noted under "Methods," with other indicated additions for the first incubation being present at twice the concentration in the second incubation.

### RESULTS

Ovine brain glutamine synthetase was inactivated by incubation with NAD and NAD-arginine ADP-ribosyltransferase (Table II). ADP-ribose and nicotinamide, 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 MgATP, previously shown to block chemical inactivation of synthetase by arginine-specific reagents (15), reduced the rate of inactivation of synthetase by transferase and NAD (Fig. 2 and...
Table III). The transferase-catalyzed inactivation of glutamine synthetase was associated with the transfer of $^{32}$P ADP-ribose from $^{32}$P NAD to the enzyme. Maximal inhibition of glutamine synthetase activity of 90% resulted after incubation of this enzyme with transferase for ~1 h, was associated with transfer of $\sim 0.89 \pm 0.07$ mol of ADP-ribose/mol of glutamine synthetase. The ratio of moles of $^{32}$P ADP-ribose/mol of glutamine synthetase to percentage inactivation was $\sim 0.99$. Glutamine synthetase purified from chicken heart was also inactivated by the transferase in an NAD-dependent reaction (Fig. 3). ADP-ribose and nicotinamide could not replace NAD (Table IV). Inhibition of the chicken heart enzyme by transferase was maximal by 1 h (Fig. 4) and was dependent on that amount of transferase 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-riboseylation (data not shown). In the presence of $^{32}$P NAD, $0.60 \pm 0.03$ mol of ADP-ribose was transferred per mol of glutamine synthetase, resulting in a 95% inactivation of the enzyme. The ratio of moles of $^{32}$P 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 $\sim 1.4$ 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 ADP-ribosyltransferase, it was observed that ADP-ribosylation 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 transferase (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 transferase. With glutamine synthetases from both sources, it appeared that inactivation resulted from the transfer of $\sim 1$ mol of ADP-ribose to 1 mol of enzyme; the reaction thus appeared to be specific.

The NAD:arginine ADP-ribosyltransferase may be used as a reagent to catalyze the covalent modification in both pure protein and tissue homogenate of arginine residues. The ADP-riboseyl(arginine) protein bond is relatively stable in acid and at physiological pH (22). In tissue homogenates, enzyme(s) responsible for degradation of the ADP-riboseyl(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 ribosyl(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 ribose(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 transferase-catalyzed reaction can be specific for certain arginine residues. Of the 25 arginine residues in ovine brain glutamine synthetase, the erythrocyte transferase and phenylglyoxyxal selectively modified that residue critical for enzymatic activity. It is clear from model studies on the transferase-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 transferase-catalyzed reaction; agmatine and arginine methyl ester were more ef-

Fig. 2. Effect of MgATP on the rate of inactivation of ovine brain glutamine synthetase. Ovine brain glutamine synthetase (40.3 µg) was incubated in 6.7% propylene glycol, 10 mM potassium phosphate (pH 7.0) and in the presence or absence of ADP-ribosyltransferase (41.9 milliunits), 1 mM NAD, 20 mM MgCl2, 15 mM ATP in a total volume of 0.6 ml. At the indicated times, 20 µl were transferred to a standard 200-µl assay mixture. The assay was run in quadruplicate for 20 min at 30°C as noted under "Methods." Glutamine synthetase activity is plotted as a percentage of that of samples incubated for the same period without NAD or transferase. ▲, + MgATP, NAD; ○, + NAD.

2 J. Moss and S. J. Stanley, unpublished data.
2 M. R. Purnell and W. R. Kidwell, unpublished data.
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 on 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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**TABLE IV**

*Inactivation of chicken heart glutamine synthetase by ADP-ribosyltransferase.*

| Additions       | MgATP | Glutamine synthetase activity (nMol·min⁻¹)* | Transferase + Transferase |
|-----------------|-------|--------------------------------------------|--------------------------|
| None            | 0     | 7.6                                        | 7.4                      |
| NAD (1 mM)      | 0     | 7.5                                        | 7.4                      |
| NAD, agmatine   | 0     | 6.5                                        | 6.9                      |
| Aminagmatine (20 mM) | 0 | 6.6                                        | 6.8                      |
| Nicotinamide (1 mM) | 0 | 7.4                                        | 7.4                      |
| ADP-ribose (1 mM) | 0 | 7.4                                        | 7.4                      |

![Fig. 3 (left). Effect of NAD on the inactivation of chicken heart glutamine synthetase by the ADP-ribosyltransferase.](image)

Chicken heart glutamine synthetase (12.2 milliunits) was incubated for 1 h at 30 °C in the presence of 10 mM Tricine (pH 7.6), 5% propylene glycol, 10 mM potassium phosphate, 200 mM NaCl, ADP-ribosyltransferase (8.19 milliunits), and the indicated concentrations of NAD (final volume, 0.1 ml). The synthetase reaction was initiated by the addition of 0.1 ml of mixture to bring the concentrations of reagents to that noted under "Methods." All assays were run in quadruplicate.

![Fig. 4 (center). Time course of inhibition of chicken heart glutamine synthetase by transferase.](image)

![Fig. 5 (right). Effect of NAD:arginine ADP-ribosyltransferase on the NAD-dependent inactivation of chicken heart glutamine synthetase.](image)
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