# Selective Probing of ADP-Ribosylation Reactions with Oxidized 2'-Deoxy-nicotinamide Adenine Dinucleotide†

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ABSTRACT: A homogeneous preparation of an arginine-specific mono(ADP-ribosyl)transferase from turkey erythrocytes effectively utilized 2'-deoxy-$NAD^+$ for the 2'-deoxy(ADP-ribose) modification of arginine methyl ester with an apparent $K_m$ of 27.2 $\mu$M and a $V_{max}$ of 36.4 $\mu$mol $min^{-1}$ (mg of protein)$^{-1}$. The adduct formed was also used as a substrate by an avian erythrocyte arginine(ADP-ribose)-specific hydrolase that generated free 2'-deoxy(ADP-ribose). In contrast, 2'-deoxy-$NAD^+$ was not a substrate in the initiation or elongation reaction catalyzed by highly purified poly(ADP-ribose) polymerase from calf thymus. However, 2'-deoxy-$NAD^+$ was a potent noncompetitive inhibitor of $NAD^+$ in the elongation reaction catalyzed by the polymerase, with an apparent $K_i$ of 32 $\mu$M. These results indicate that 2'-deoxy-$NAD^+$ may be utilized to specifically identify protein acceptors for endogenous mono(ADP-ribosyl)transferases in complex biological systems that may contain a high activity of poly(ADP-ribose) polymerase, i.e., cell nuclei preparations.

The posttranslational covalent modification of proteins by ADP-ribosylation has been shown to play a critical role in the regulation of several metabolic pathways (Moss & Vaughan, 1988; Mandel et al., 1982; Ueda & Hayaishi, 1985; Althaus & Richter, 1987). These reactions are catalyzed by two different classes of enzymes, which utilize $\beta$-$NAD^+$ as substrate. Mono(ADP-ribosyl)transferases catalyze the mono(ADP-ribosylation) of proteins (Vaughan & Moss, 1981; Ueda & Hayaishi, 1985). Conversely, poly(adenosine diphosphate ribose) (ADP-ribose)[1] polymerase (EC 2.4.2.30), a DNA-dependent enzyme, catalyzes the initial ADP-ribosylation and subsequent chain elongation of specific chromatin proteins (Ueda & Hayaishi, 1985; Althaus & Richter, 1987). Mono(ADP-ribosyl)transferases have been isolated from both prokaryotes (Vaughan & Moss, 1981; Ueda & Hayaishi, 1985) and eukaryotes (Moss & Vaughan, 1978; Moss et al., 1980; Yost & Moss, 1983; Tanigawa et al., 1984; Soman et al., 1984). In contrast, poly(ADP-ribose) polymerase has only been identified in higher eukaryotes (Scovassi et al., 1986).

The physiological importance of mono(ADP-ribosylation) reactions has been best documented for some bacterial toxins that possess mono(ADP-ribosyl)transferase activity and alter the physiological function of critical enzymes and proteins of mammalian cells, i.e., *Pseudomonas* toxin (Iglewski & Kabat, 1975), diphtheria toxin (Collier, 1975), cholera toxin (Moss & Vaughan, 1981), *Escherichia coli* enterotoxin (Moss & Richardson, 1978), pertussis toxin (Katada & Ui, 1982), and *Botulinum* C-2 toxin (Aktories et al., 1986). In addition, nitrogenase activity in the photosynthetic bacterium *Rhodosporillum rubrum* has also been shown to be regulated by an arginine-specific mono(ADP-ribosylation) cycle (Pope et al., 1986; Lowery et al., 1986). Arginine-specific mono(ADP-ribosyl)transferases have also been found in animal tissues (West & Moss, 1986; Tanigawa et al., 1984). However, no specific function has been ascribed to them.

In contrast, there is compelling evidence linking poly(ADP-ribose) biosynthesis with cellular responses to DNA damage (Shall, 1984; Berger, 1985). For example, poly(ADP-ribosylation) of chromatin proteins has been shown to be required for efficient DNA excision repair (Durkacz et al., 1980). This reaction is also believed to be involved in other cellular processes that involve changes in chromatin structure, such as DNA replication, cellular differentiation, and tumorigenesis [for a review, see Althaus and Richter (1987)]. The biosynthesis of highly complex polymers of ADP-ribose in vivo (Alvarez-Gonzalez & Jacobson, 1987) may be a general mechanism for the modulation of chromatin function (Poirier et al., 1982; DeMurcia et al., 1986).

All of the ADP-ribosyltransferases described to date have identical substrate stereospecificity in which the $\beta$ configuration of the anomeric carbon of $NAD^+$ is converted to the $\alpha$ configuration found in the product (Ferro & Oppenheimer, 1978; Moss et al., 1979). Apart from the spectrum of acceptor sites, no differences in substrate specificity that would allow dissection of these activities in complex biological systems have been detected between these enzymes. The present study identifies a differential behavior of an avian erythrocyte $NAD^+$:arginine mono(ADP-ribosyl)transferase and calf thy-

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[1] Abbreviations: 2'-dADP-ribose, 2'-deoxyadenosine diphosphate ribose; ADP-ribose, adenosine diphosphate ribose; $\beta$-$NMN^+$, $\beta$-nicotinamide mononucleotide; 2'-dATP, 2'-deoxyadenosine 5'-triphosphate; 2'-$dNAD^+$, 2'-deoxy-nicotinamide adenine dinucleotide; TCA, trichloroacetic acid; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; DTT, dithiothreitol; HPLC, high-pressure liquid chromatography; SAX-HPLC, strong anion exchange-high-pressure liquid chromatography; $\varepsilon$RAdo, ethenoribosyladenosine; BAP, bacterial alkaline phosphatase.

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mus poly(ADP-ribose) polymerase toward 2′-dNAD$^+$ as a substrate.

## Experimental Procedures

*Materials.* Arginine methyl ester, NMN$^+$, 2′-dATP, ovalbumin, inorganic pyrophosphatase, and bacterial alkaline phosphatase (BAP), type II-S, were obtained from Sigma Chemical Co. (St. Louis, MO). Affi-Gel 601 was purchased from Bio-Rad (Richmond, CA). The supplier of NMN$^+$ adenyltransferase (NAD$^+$ pyrophosphorylase) from hog liver was Boehringer (Mannheim, West Germany). 3-Aminobenzamide was supplied by TCI (Tokyo, Japan). The Partisil 10-SAX column (250 × 4.5 mm i.d.) and packing material were obtained from Whatman Chemical-Separation, Inc. (Clifton, NJ). Radiolabeled [$\alpha$-$^{32}$P]-2′-dATP (<3000 Ci/mmol) was purchased from Amersham International (Buckinghamshire, England). [*adenosyl*-$^{14}$C(U)]NAD$^+$ (601 mCi/mmol) was obtained from New England Nuclear (Boston, MA). All other chemicals used were reagent grade.

*Synthesis and Purification of 2′-dNAD$^+$.* Radiolabeled [$^{32}$P]-2′-dNAD was synthesized by incubating for 2 h at 37 °C a mixture containing 100 mM glycylglycine buffer (pH 7.4), 10 mM $MgCl_2$, 3.0 mM NMN$^+$, 500 $\mu$M [$\alpha$-$^{32}$P]-2′-dATP (2 Ci/mmol), 3 units of inorganic pyrophosphatase, and 0.2 unit of NAD$^+$ pyrophosphorylase (final volume 1 mL). The reaction was stopped by placing the mixture at 0 °C, and the solution was immediately loaded on a 1-mL column of Affi-Gel 601 to eliminate unreacted 2′-dATP (Alvarez-Gonzalez et al., 1983). The column eluate was lyophilized to dryness and incubated with 3 units of BAP for 1 h at 37 °C in 1 mL of 20 mM Tris-HCl buffer, pH 8.0. The incubation mixture was then analyzed by HPLC on a Partisil 10-SAX column (250 × 4.5 mm i.d.), with a guard column (75 × 2.5 mm i.d.) utilizing a low-salt buffer system at a flow rate of 1 mL/min. The 254-nm absorbing peak containing the radiolabeled 2′-dNAD$^+$ was collected and desalted on Affi-Gel 601 as indicated above. The boronate-purified material was lyophilized to dryness, redissolved in a small volume of water, and stored at −20 °C. Typically, about 30% of the initial radiolabel was recovered as pure 2′-dNAD$^+$.

*Purification and Assay of Poly(ADP-ribose) Polymerase.* The DNA-independent enzyme was purified from calf thymus as described by Niedergang et al. (1979). The DNA-dependent enzyme was also purified from calf thymus by the procedure of Zahradka and Ebisuzaki (1984). Poly(ADP-ribose) polymerase activity was determined by measuring the amount of radiolabeled [*adenosyl*-$^{14}$C(U)]NAD$^+$ incorporated into 20% (w/v) TCA-precipitable material. A 100-$\mu$L mixture containing 25 mM Tris-HCl buffer, pH 8.0, 10 mM $MgCl_2$, 1 mM DTT, 2.17 pmol of pure enzyme, and either 10 $\mu$M [$^{14}$C]NAD$^+$ (400 cpm/pmol) or [*adenylate*-$^{32}$P]-2′-dNAD$^+$ was incubated at 25 °C for the specified times. All incubations were minimally carried out in duplicate. The reaction was stopped by the addition of 900 $\mu$L of 20% (w/v) TCA and 100 $\mu$L of 1% bovine serum albumin at 0 °C. The solution was centrifuged at 15 000 rpm for 15 min, and the acid-insoluble material was rinsed three times with 20% (w/v) TCA and twice with ether. An aliquot of this solution was utilized for measuring, by liquid scintillation counting, total radioactivity incorporated.

*Purification and Assay of Mono(ADP-ribosyl)transferase.* Mono(ADP-ribosyl)transferase from turkey erythrocytes was purified to homogeneity as described by Moss et al. (1980). Incubations for enzyme activity measurements were carried out at 37 °C, in triplicate, in mixtures of 300 $\mu$L containing 50 mM potassium phosphate buffer, pH 7.5, 75 mM arginine methyl ester, 0.3 mg of ovalbumin, 0.1 milliunit of pure enzyme, and either 32.4 $\mu$M $\beta$-NAD$^+$ or 32 $\mu$M [*adenylate*-$^{32}$P]-2′-dNAD$^+$ as the substrate. The reaction was stopped at the specified times by diluting 1:10 with 50 mM potassium phosphate buffer, pH 4.7, at 0 °C. The nucleotide content was then determined by SAX–HPLC, with 50 mM potassium phosphate buffer, pH 4.7, as the solvent system at a flow rate of 1 mL/min (Alvarez-Gonzalez et al., 1986). The course of the reaction was monitored by measuring both the disappearance of the $\beta$-NAD$^+$ peak and the appearance of ADP-ribosylated arginine methyl ester. For experiments with the ADP-ribosylarginine hydrolase, arginine methyl ester modified with either ADP-ribose or 2′-dADP-ribose was purified by collecting the peak from the Partisil 10-SAX column and subsequent affinity chromatography on Affi-Gel 601.

Table I: Binding of 2′-Deoxy-ATP and 2′-Deoxy-NAD$^+$ to Affi-Gel 601[a]

| nucleotide | radioactivity (cpm)[b] total | bound | % bound |
|---|---|---|---|
| 2′-dATP | 3 888 000 | 5 170 | 0.13 |
| 2′-dNAD$^+$ | 91 660 | 76 810 | 83.8 |

[a] $^{32}$P-Radiolabeled deoxynucleotides were chromatographed on a column containing 1 mL of Affi-Gel 601 as described by Alvarez-Gonzalez et al. (1983). [b] The specific radioactivities of [$^{32}$P]-2′-dATP and [$^{32}$P]-2′-dNAD$^+$ were 3000 and 2 Ci/mmol, respectively.

*Purification and Assay of ADP-ribosylarginine Hydrolase.* The enzyme was partially purified from turkey erythrocytes as described by Moss et al. (1986). Hydrolase activity was measured in a total volume of 200 $\mu$L containing 50 mM potassium phosphate buffer, pH 7.5, 10 mM $MgCl_2$, 5 mM DTT, and either 40 $\mu$M ADP-ribosylarginine methyl ester or 50 $\mu$M 5′-dADP-ribosylarginine methyl ester. The reaction was initiated by the addition of 58 milliunits of the enzyme at 30 °C. At different times of incubation, the reaction was stopped by a 10-fold dilution with 50 mM potassium phosphate buffer, pH 4.7, at 0 °C. The samples were then analyzed by SAX–HPLC, with 50 mM potassium phosphate buffer, pH 4.7, as the solvent system at a flow rate of 1 mL/min. The course of the reaction was followed by simultaneous monitoring of the disappearance of the substrate and the formation of products. Assays were performed in duplicate.

## Results

*Synthesis and Purification of 2′-dNAD$^+$.* We have used NMN$^+$ adenyltransferase (EC 2.7.7.18) to enzymatically synthesize [$^{32}$P]-2′-dNAD$^+$ from [$\alpha$-$^{32}$P]-2′-dATP with a yield of approximately 30%. To remove residual [$\alpha$-$^{32}$P]-2′-dATP, the incubation mixture was subjected to affinity chromatography on Affi-Gel 601 under conditions that allow quantitative binding of nucleosides and nucleotides to immobilized boronates (Alvarez-Gonzalez et al., 1983). Table I shows that while 2′-dNAD$^+$ efficiently bound to the boronate gel, 2′-dATP, which lacks the required vicinal *cis*-diol group, did not. The mixture was then treated to eliminate residual $\beta$-NMN$^+$, which, although not radiolabeled, also bound to Affi-Gel 601. Removal of this contaminant was facilitated by subjecting these nucleotides to BAP treatment, as indicated under Experimental Procedures, to convert NMN$^+$ to nicotinamide ribose, which was not retained by the Partisil 10-SAX column. Fractions containing the dinucleotide were collected, concentrated on Affi-Gel 601, lyophilized, and reanalyzed by SAX–HPLC. This material represented pure 2′-dNAD$^+$, and 100% of the $^{32}$P radiolabel coeluted with this peak (not shown).

*Utilization of 2′-dNAD$^+$ as a Substrate for Mono(ADP-ribosyl)transferases.* The ability of mono(ADP-ribosyl)-

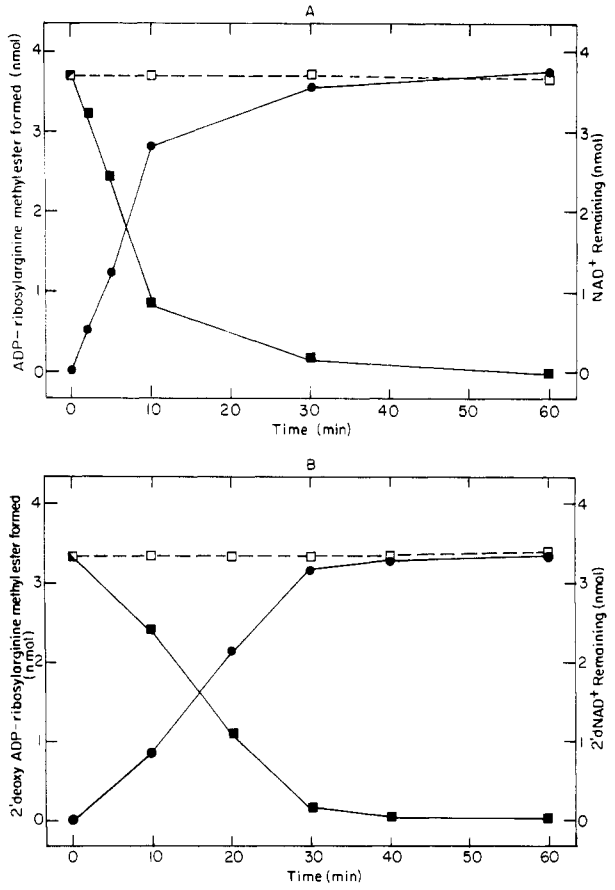


FIGURE 1: Analysis of 2′-dNAD$^+$ as a substrate for purified mono(ADP-ribosyl)transferases. (A) Time-dependent utilization of $\beta$-NAD$^+$ (■) for the synthesis of mono(ADP-ribosyl)arginine methyl ester (●) by an NAD$^+$:arginine mono(ADP-ribosyl)transferase from avian erythrocytes. Enzyme activity was measured by concomitant monitoring by SAX–HPLC of substrate and product at different times of incubation as described under Experimental Procedures. (B) Duplicate incubation reactions in which 2′-dNAD$^+$ (■) was utilized as a substrate for the synthesis of 2′-dADP-ribosylarginine methyl ester (●) with an arginine-specific mono(ADP-ribosyl)transferase. (□) Amount of substrate remaining following incubation with boiled enzyme.

transferases to utilize 2′-dNAD$^+$ as a substrate was examined with a homogeneous preparation of an arginine-specific enzyme from turkey erythrocytes (Moss et al., 1980). We determined the rate of the reaction by measuring the amount of ADP-ribosylated acceptor formed, utilizing SAX–HPLC as specified under Experimental Procedures. Figure 1A shows the temporal relationship between the amount of $\beta$-NAD$^+$ consumed and the appearance of the ADP-ribosylated amino acid. Previous boiling of the enzyme totally prevented the reaction. We have utilized the same method to monitor the reaction using 2′-dNAD$^+$ as the "ADP-ribose" donor. The retention times of the substrates NAD$^+$ and 2′-dNAD$^+$ as well as the mono(ADP-ribosylated) and mono(2′-dADP-ribosylated) arginine methyl ester were 7.11, 9.22, 6.14, and 6.88 min, respectively. As shown in Figure 1B, the mono(ADP-ribosyl)transferase efficiently utilized 2′-dNAD$^+$ to modify arginine methyl ester. A more detailed kinetic analysis indicated that this transferase utilized 2′-dNAD$^+$ as a substrate, with a $K_m$ of 27.2 $\mu$M and a $V_{max}$ of 36.4 $\mu$mol min$^{-1}$ (mg of protein)$^{-1}$.

Further proof that the product of the reaction indicated above corresponded to mono(2′-dADP-ribosyl)arginine methyl ester is presented in Figure 2. We have purified this putative material, as indicated under Experimental Procedures, and

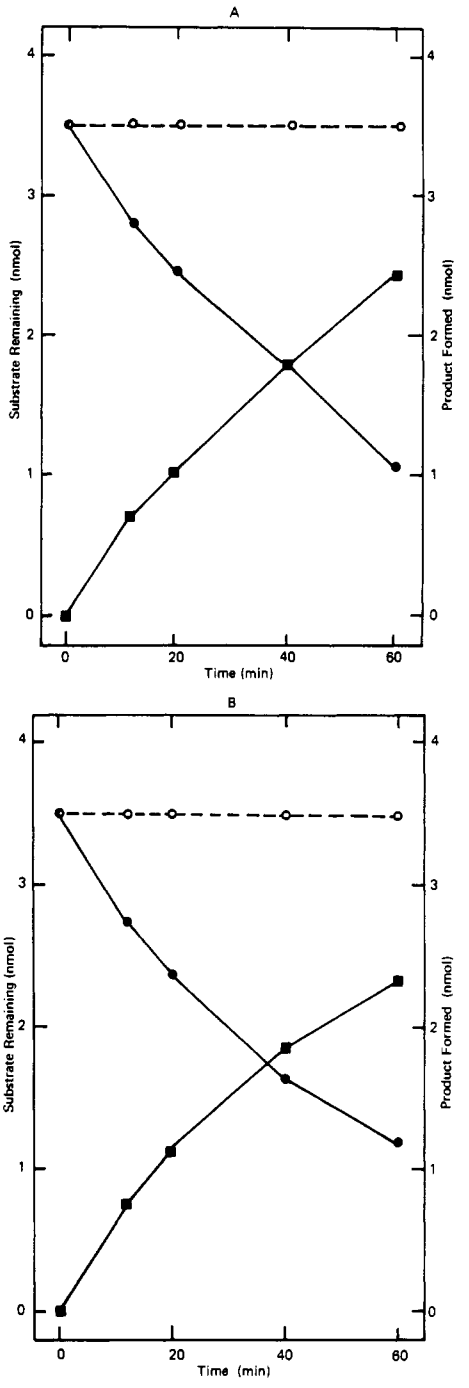


FIGURE 2: Analysis of 2′-dADP-ribosylarginine methyl ester as a substrate for purified ADP-ribosylarginine-specific hydrolase. (A) Time-dependent degradation of ADP-ribosylarginine methyl ester (●) to ADP-ribose (■) following incubation at 30 °C with ADP-ribosylarginine hydrolase as indicated under Experimental Procedures. (B) Average of duplicate experiments in which the putative 2′-dADP-ribosylarginine methyl ester (●) was utilized as a hydrolase substrate, leading to the formation of 2′-dADP-ribose (■). (○) Amount of adduct remaining following incubation with boiled enzyme.

utilized it as a substrate for an ADP-ribosylarginine hydrolase (Moss et al., 1986). In this assay, SAX–HPLC was also used to monitor the time course of the reaction. The retention time of the products of this reaction, either ADP-ribose or 2′-dADP-ribose, corresponded to 15.04 and 15.42 min, respectively. Figure 2A shows that incubation of the hydrolase with ADP-ribosylarginine methyl ester at 30 °C resulted in the formation of free ADP-ribose in a time-dependent fashion. The reaction did not reach 100% completion, presumably because the free ADP-ribose formed inhibited the enzyme

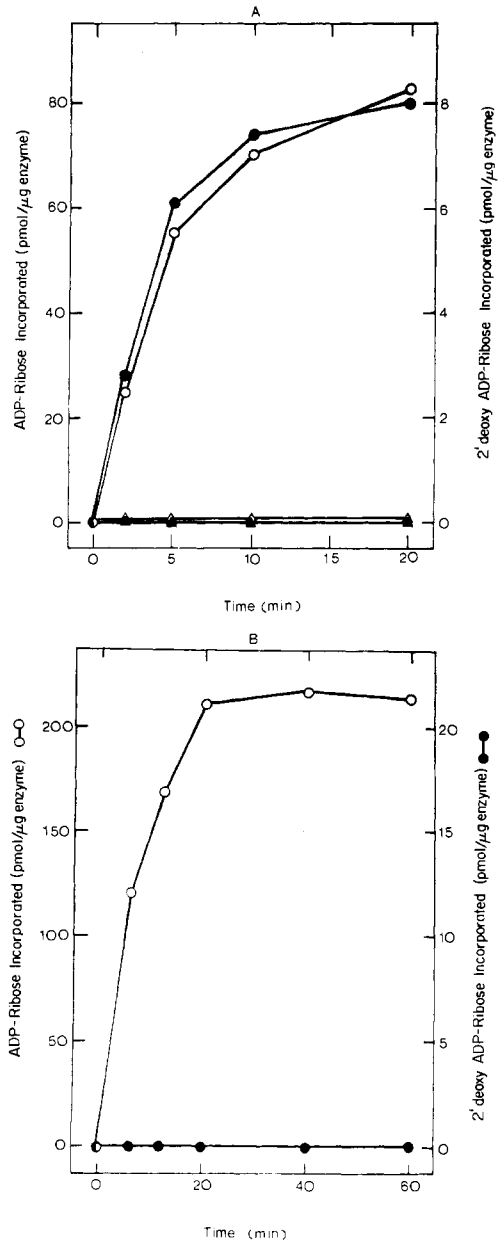


FIGURE 3: Analysis of [*adenylate*-$^{32}$P]-2′-dNAD$^+$ as a substrate for the initiation reaction catalyzed by poly(ADP-ribose) polymerase. (A) Incorporation of either labeled ADP-ribose from [$^{14}$C]NAD$^+$ (○, ●) or labeled 2′-dADP-ribose from [$^{32}$P]-2′-dNAD$^+$ (△, ▲) into acid-precipitable material following incubation with highly purified poly(ADP-ribose) polymerase at 25 °C in the presence (●, ▲) or absence (○, △) of histone H1. (B) Specific modification of histone H1 by poly(ADP-ribose) polymerase under conditions where substantial enzyme automodification was limited by omitting $Mg^{2+}$ ions from the incubation mixture (Tanaka et al., 1979).

activity (ADP-ribose, at micromolar concentrations, is a competitive inhibitor of the hydrolase). Alternatively, the substrate might have anomerized to yield a mixture of the $\alpha$ and $\beta$ derivatives, and only the $\alpha$ anomer is a hydrolase substrate (Moss et al., 1986). As shown in Figure 2B, treatment of the putative mono(2′-dADP-ribosyl)arginine methyl ester with the purified ADP-ribosylarginine hydrolase resulted in the generation of 2′-dADP-ribose. This indicated that the adduct formed with the mono(ADP-ribosyl)transferase was in fact 2′-dADP-ribosylarginine methyl ester. Boiling of the hydrolase prior to the assay totally prevented the hydrolysis of the adducts (Figure 2A,B).

*Utilization of 2′-dNAD$^+$ as a Substrate for Poly(ADP-ribose) Polymerase.* As a prerequisite to test whether 2′-dNAD$^+$ served as a substrate for the initiation reaction catalyzed by purified poly(ADP-ribose) polymerase, we first determined whether or not the enzyme preparation contained endogenously bound polymers. Polymeric residues (ξRAdo) were not detected in 17 pmol of enzyme protein under conditions that allow quantitative detection of less than 1 pmol of polymeric ADP-ribose (Jacobson et al., 1984). Figure 3A shows that while DNA-independent poly(ADP-ribose) polymerase purified from calf thymus catalyzed the formation of protein-bound poly([$^{14}$C]ADP-ribose) in the presence or absence of histone H1 at 10 $\mu$M [$^{14}$C]NAD$^+$, no incorporation into TCA-precipitable material was observed when radiolabeled [*adenylate*-$^{32}$P]-2′-dNAD$^+$ was used as a substrate. In fact, as shown on Figure 3B, the same negative results were obtained when the reaction was carried out in the absence of $Mg^{2+}$, conditions under which preferential histone H1 modification has been shown (Tanaka et al., 1979). Identical results were obtained with a DNA-dependent preparation of poly(ADP-ribose) polymerase purified from calf thymus by the procedure of Zahradka and Ebisuzaki (1984) (not shown).

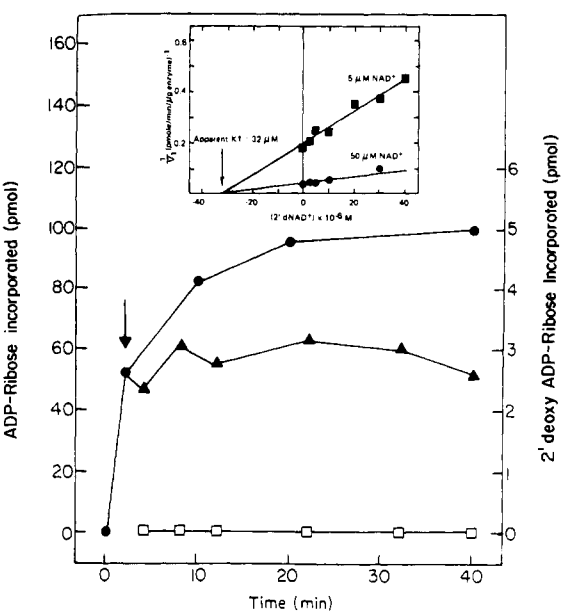


FIGURE 4: Analysis of [*adenylate*-$^{32}$P]-2′-dNAD$^+$ as a substrate for the elongation reaction catalyzed by poly(ADP-ribose) polymerase. The arrow indicates the addition of 80 $\mu$M 2′-dNAD$^+$ to an incubation mixture containing 10 $\mu$M NAD$^+$, under conditions favoring the automodification of the enzyme. (□) Radiolabeled 2′-dADP-ribose incorporated from [$^{32}$P]-2′-dNAD$^+$ into 20% TCA-precipitable material. Incorporation of radiolabeled ADP-ribose from [$^{14}$C]NAD$^+$ into acid-insoluble material with (▲) and without (●) the addition of 2′-dNAD$^+$. Inset: Dixon plot showing the inhibition of the automodification of poly(ADP-ribose) polymerase by increasing amounts of 2′-dNAD$^+$ at two constant concentrations of NAD$^+$, 5 $\mu$M (■) and 50 $\mu$M (●), respectively.

We have also examined 2′-dNAD$^+$ as a substrate for the elongation reaction catalyzed by the polymerase by adding 80 $\mu$M [*adenylate*-$^{32}$P]-2′-dNAD$^+$ after 2 min of incubation of the enzyme with 10 $\mu$M [$^{14}$C]NAD$^+$. Under these conditions, no incorporation of $^{32}$P radiolabel into acid-insoluble material was observed, as shown in Figure 4. This result is consistent with the conclusion that 2′-dNAD$^+$ is not a substrate for the elongation reaction catalyzed by poly(ADP-ribose) polymerase.

*Utilization of 2′-dNAD$^+$ as an Inhibitor of the Elongation Reaction Catalyzed by Poly(ADP-ribose) Polymerase.* Addition of 2′-dNAD$^+$ to a polymerase automodification assay abruptly stopped the incorporation of $^{14}$C-radiolabeled NAD$^+$ into acid-insoluble material, as shown in Figure 4. These observations were consistent with the hypothesis that 2′-

dNAD$^+$ was a potent inhibitor of the elongation reaction. Careful kinetic analysis of the mode of inhibition of poly(ADP-ribose) biosynthesis by 2′-dNAD$^+$ as described by Dixon (1953) indicated that 2′-dNAD$^+$ was a potent noncompetitive inhibitor of the polymerase with an apparent $K_i$ of 32 $\mu$M (inset of Figure 4).

## Discussion

In the late 1970s, it was reported that several chromatin proteins could be 2′-dADP-ribosylated by incubating a crude preparation of rat liver nuclei with radiolabeled 2′-dNAD$^+$ (Suhadolnik et al., 1977; Lichtenwalner & Suhadolnik, 1979). These observations have since been interpreted to indicate that 2′-dNAD$^+$ is utilized as a substrate by poly(ADP-ribose) polymerase (Suhadolnik, 1982). It has also recently been reported by Ikejima et al. (1987) that poly(ADP-ribose) polymerase is 2′-dADP-ribosylated following incubation of a HeLa cell lysate with 2′-dNAD$^+$. However, the results obtained in this study clearly demonstrate that 2′-dNAD$^+$ is not a substrate for highly purified poly(ADP-ribose) polymerase from calf thymus, as shown in Figures 3 and 4. In fact, we found that 2′-dNAD$^+$ was instead a potent noncompetitive inhibitor of poly(ADP-ribose) elongation (Figure 4). This mode of inhibition indicates that 2′-dNAD$^+$ is not interfering with the NAD$^+$ binding site of the enzyme and suggests that it may be blocking the binding of the other substrate of the polymerase, i.e., the AMP terminus of the growing polymer. Further experiments are currently in progress to study this possibility. It is also important to note that while Ikejima et al. (1987) suggested that 2′-dNAD$^+$ was a competitive inhibitor of poly(ADP-ribose) biosynthesis, their conclusion was based on the utilization of permeabilized cells as the enzyme source. Under these conditions, the presence of multiple ADP-ribosyltransferase activities complicates the interpretation of data related to the kinetics of the polymerase. The 2′-dADP-ribose modification of chromatin proteins with crude nuclei preparations and permeabilized cells with 2′-dNAD$^+$ as the substrate (Suhadolnik, 1982; Ikejima et al., 1987) may have resulted from either (i) the nonenzymatic ADP-ribosylation of proteins at high substrate concentrations (Hilz et al., 1984) or (ii) the activity of a mono(ADP-ribosyl)-transferase. We have examined this second possibility by testing the ability of a highly purified NAD$^+$:arginine mono(ADP-ribosyl)transferase to use 2′-dNAD$^+$ as a substrate (Moss et al., 1980). Positive results obtained with these experiments (Figure 1) supported the view that our enzymatically synthesized 2′-dNAD$^+$ truly corresponded to the $\beta$ isomer, which is the only substrate for both mono(ADP-ribosyl)-transferases (Moss et al., 1980) and poly(ADP-ribose) polymerase (Ferro & Oppenheimer, 1978). Furthermore, while the incubation of pure poly(ADP-ribose) polymerase with $^{32}$P-labeled 2′-dNAD$^+$ in the presence of active calf thymus DNA does not produce automodified enzyme, the addition of the arginine-specific mono(ADP-ribosyl)transferase from turkey erythrocytes, under similar conditions, results in the mono(2′-dADP-ribosylation) of poly(ADP-ribose) polymerase, presumably at an arginine residue (R. Alvarez-Gonzalez, unpublished observations). Therefore, these results are consistent with the conclusion that a mono(ADP-ribosyl)-transferase rather than poly(ADP-ribose) polymerase was responsible for the 2′-dADP-ribosylation of chromatin proteins shown by Lichenwalner and Suhadolnik (1979) as well as by Ikejima et al. (1987). The utilization of 2′-dNAD$^+$ may prove helpful in selectively identifying protein acceptors of mono(ADP-ribosyl)transferases in complex biological systems where contamination of poly(ADP-ribose) polymerase is significant.

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# Carbon Monoxide Oxygenase Activity of Cytochrome $cd_1$[†]

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ABSTRACT: Cytochrome $cd_1$ from the denitrifying bacterium *Pseudomonas aeruginosa* catalyzes the oxygenation of carbon monoxide by dioxygen. A minimum estimate of the turnover number for this activity is 7 mol of carbon dioxide produced per hour per mole of cytochrome subunit at 30 °C and pH 7. The reaction is 98% inhibited by 2.5 mM cyanide, but catalase has no effect. The reaction accounts for the unusual reduction of ferric cytochrome in the presence of carbon monoxide, but no additional reducing agent. The reaction is independent of the steady-state oxidation level of the cytochrome during turnover. Under anaerobic conditions, ferricyanide plus water may substitute for dioxygen as the source of oxidizing equivalents and atomic oxygen.

Cytochrome $cd_1$ is a dissimilatory nitrite reductase found in chemoautotrophic, denitrifying bacteria. It contains one heme *c* and one heme $d_1$ per polypeptide of molecular weight 60000. Two subunits form the active dimer (Kuronen et al., 1975). In addition to nitrite reduction, it can catalyze the reduction of oxygen to water (Timkovich & Robinson, 1979), although only nitrite reduction is its physiological function (Yamanaka, 1964). The heme *c* is known to be low spin with strong evidence of six-coordinate bonds in both ferric and ferrous oxidation states. The heme $d_1$ is unusual in that in the ferric state it is low spin and presumably six coordinated, but in the ferrous state it is high spin and presumably five-coordinated (Gudat et al., 1973; Orii et al., 1977; Vickery et al., 1978; Walsh et al., 1979; Timkovich & Cork, 1982, 1983; Timkovich et al., 1985; Sutherland et al., 1986).

When anaerobic, ferric cyt $cd_1$[1] is exposed to carbon monoxide, the iron centers become ferrous within minutes to hours, depending upon conditions. This behavior has been reported by independent groups (Barber et al., 1976; Vickery et al., 1978) and has been called "autoreduction", because no common or obvious chemical reductant has been added. CO is not generally recognized as a reducing agent for ferric iron, and in the previous reports no attempt was made to identify the reductant source. Reduction in the presence of CO is a property also demonstrated by purified mammalian cytochrome oxidase, cyt $aa_3$ (Tzagoloff & Wharton, 1965; Greenwood et al., 1974). Tzagoloff and Wharton (1965) originally demonstrated that $CO_2$ was a product of the cyt $aa_3$ reaction, while recently Young and Caughey (1986) investigate the catalytic rate of oxidation of CO to $CO_2$. The enzymatic activity was called oxygenase activity, because the available data indicated that the added oxygen atom came from $O_2$. Here we demonstrated that cyt $cd_1$ also possesses strong CO oxygenase activity.

## Materials and Methods

*Pseudomonas aeruginosa* (ATCC 19428) was cultured and cyt $cd_1$ purified as described previously (Timkovich & Cork, 1982). It was precipitated by the addition of solid ammonium sulfate to 90% saturation, and the pellet was recovered after centrifugation. Protein was redissolved in 0.1 M potassium phosphate buffer with 5 mM EDTA, pH 7.0, that had been sterilized by passage through a Nalgene sterilizing filter unit. Individual samples of 0.5 mL were frozen in liquid nitrogen until use. Protein concentration was determined with standard extinction coefficients (Silvestrini et al., 1979) and will be reported as the concentration of subunits. $^{13}CO$, 99.4 atom % $^{13}C$, was purchased from MSD Isotopes. Other gases of research purity were purchased from Matheson.

Special cells were constructed to measure the infrared spectrum of the gas phase above an aqueous enzyme solution. Two types were employed that differed only in the type of seal against the outside atmosphere. In the first type a tube 2.5 cm long with 1-cm i.d. was fused at a right angle to a tube 10 cm long with 2.3-cm i.d. and 2.5-cm o.d.. The smaller side tube was fit with a standard rubber septum where gases and reagents could be added by a needle. The second type was designed to ensure a higher integrity seal. The small side tube

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[1] Abbreviations: cyt, cytochrome; $p_{O_2}$, partial pressure of oxygen; $p_{CO}$, partial pressure of carbon monoxide; GC/MS, gas chromatography–mass spectrometry.

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