Covalent Binding of 4-Carbamoylbenzenediazonium Chloride to Deoxyguanine Bases of DNA Resulting in Apparent Irreversible Inhibition of Poly(adenosine diphosphoribose) Polymerase at the Nicotinamide Binding Site†

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Received September 25, 1986; Revised Manuscript Received December 12, 1986

ABSTRACT: The poly(adenosine diphosphoribose) polymerase activity of isolated liver nuclei was inhibited by 4-carbamoylbenzenediazonium chloride, referred to as 4-diazoniobenzamide, an effect that was dependent on the time of incubation and the concentration of the diazonium compound, with inhibition following first-order kinetics. The inhibition was not reversed by reisolation of nuclei and centrifugal washing, whereas the inhibition by benzamide or 4-aminobenzamide was completely reversible under these conditions. Simultaneous incubation of 4-diazoniobenzamide with benzamide prevented enzyme inhibition. The 4-diazoniobenzoic acid analogue was not inhibitory. The mechanism of action of 4-diazoniobenzamide was traced to a specific covalent binding to dGMP of DNA to form N^2-[(4-carbamoylphenyl)azo]-2′-deoxyguanosine 5′-monophosphate. Coenzymic DNA, by tight association with the polymerase protein, fixes the $-C(O)NH_2$ moiety of the adduct at the nicotinamide-binding site of the enzyme.

The coenzymic requirement for DNA in the poly(ADP-ribose)[1] polymerase (EC 2.4.2.30) catalyzed synthesis of protein-bound polymers of ADP-ribose is well established [cf. Ueda and Hayaishi (1985) and Gaal and Pearson (1985)]. An enzyme-associated DNA species or sequence can be dissociated from the isolated enzyme (Yoshihara et al., 1978) that on a weight basis is more efficient as a "coenzyme" of the polymerase than crude thymus DNA. Several hypotheses were proposed to explain the role of DNA in poly-ADP-ribosylations. Single- and double-strand breaks of DNA in many systems increase its catalytic effectivity on poly-ADP-ribosylation (Miller, 1975; Benjamin & Gill, 1980a,b; Kawaichi et al., 1981), suggesting that the process of DNA fragmentation itself might be responsible for enzyme activation. However, the infrequent association of the enzyme protein with DNA termini (DeMurcia et al., 1983; Ittel et al., 1985) seems inconsistent with a hypothesis that correlates DNA fragmentation with increased coenzymic activity. Furthermore, a high degree of DNA fragmentation was shown to coincide with low enzymatic activity in certain cell types (Skidmore et al., 1985); thus activation of poly-ADP-ribosylations by DNA fragmentation is not universal (Jackowski & Kun, 1983). It was concluded on the basis of enzymatic experiments with synthetic oligodeoxyribonucleotides (Berger & Petzold, 1985) that small coenzymatically active DNA fragments may exist in nuclei which cannot be detected by tests suitable for the determination of DNA fragmentation.

Distinct peptide fragments were isolated from the enzyme molecule that participate in DNA and NAD^+ binding (Kameshita et al., 1986), and the experimentally observed influence of the concentration of coenzymatic DNA on the apparent K_m of NAD^+ suggests that the DNA- and NAD^+-binding sites may interact in the intact enzyme molecule (Niedergang et al., 1979). Determination of the localization of DNA- and NAD^+-binding sites of the intact enzyme is critical for the understanding of the mechanism of poly-ADP-ribosylation. We demonstrate here that covalent binding of 4-diazoniobenzamide[2] to DNA renders benzamide, which is a reversible inhibitor (Purnell & Whish, 1980), to a tightly enzyme associated species that simulates irreversible inhibition. The tight association of coenzymic DNA to the enzyme serves as an adaptor for the benzamide moiety of the DNA adduct, fixing the DNA-bound benzamide at the nicotinamide binding site of the enzyme protein. The molecular structure of the dGMP adduct of 4-diazoniobenzamide was determined, identifying the binding site of the inhibitor on DNA.

Experimental Procedures

Materials. Benzamide and 3,5-diiodo-4-aminobenzoic acid were obtained from Aldrich Chemical Co., and 4-aminobenzamide, 4-aminobenzoic acid, *N*-hydroxysuccinimide, dicyclohexylcarbodiimide, (chloroacetyl)tyrosine, sodium nitrite, NAD^+, dGMP, dAMP, dTMP, dCMP, deuterium oxide, and dimethyl sulfoxide-d_6 were purchased from Sigma Chemical Co. [^{32}P]NAD^+ was obtained from New England Nuclear and [^{14}C]dGMP from Amersham. Silica–alumina catalyst pellets were obtained from Davison Chemical Division of W. R. Grace and Co. All other reagents and solvents were of analytical grade.

† This research was supported by National Institutes of Health Grant HL27317 and Air Force Office of Scientific Research Grant AFO-SR-86-0064. Mass spectrometry was carried out at the Bio-Organic Mass Spectrometry Resource, supported by the National Institutes of Health, Division of Research Resource Grant RR01614. We are indebted to Chin-Tzu Peng (School of Pharmacy) for performing the catalytic tritiation reaction (supported by NIH Grant CA33547).

* Recipient of the Research Career Award of the U.S. Public Health Service. Correspondence should be addressed to this author.

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[1] Abbreviations: poly(ADP-ribose), poly(adenosine diphosphoribose); NAD^+, oxidized nicotinamide adenine dinucleotide; dGMP, 2′-deoxyguanosine 5′-monophosphate; dCMP, 2′-deoxycytidine 5′-monophosphate; dTMP, thymidine 5′-monophosphate; dAMP, 2′-deoxyadenosine 5′-monophosphate; HPLC, high-performance liquid chromatography; NMR, nuclear magnetic resonance; TLC, thin-layer chromatography; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride.

[2] For convenience 4-diazoniobenzamide is used throughout the text to denote 4-carbamoylbenzenediazonium chloride.

0006-2960/87/0426-2226$01.50/0

Spectroscopy. Mass spectra, both in the high-resolution electron impact mode and the liquid secondary ionization mode, were measured on a Kratos MS-50S mass spectrometer equipped with a 23-kG magnet and a postacceleration detector, NMR spectra were measured on a Varian FT-80 NMR spectrometer, and UV spectra were measured on a Perkin-Elmer 552 spectrophotometer.

HPLC. HPLC was performed with the following instrumental components: Waters Model 6001 solvent delivery pumps, Waters Model 680 gradient controller, and Hewlett-Packard Model 1040 high-speed spectrophotometric detector. HPLC eluate fractions were collected with an Isco fraction collector, and radioactivity was counted on a Beckman Model LS-3800 scintillation counter. The column employed was a Beckman-Altex analytical reversed-phase column (Ultrasphere ODS, 5 μm, 25 cm × 4.6 mm i.d.) with a precolumn packed with the same sorbent as the analytical column. Chromatography was carried out at ambient temperature. The buffer system employed was as follows: buffer A, 0.1 M potassium phosphate, pH 4.25; buffer B, 0.1 M potassium phosphate, pH 4.25, and 50% acetonitrile. Upon sample injection, elution gradient was started from 100% buffer A by using the concave gradient (controller curve 9) to 100% buffer B during 20 min. After completion of the gradient, elution was continued with 100% buffer B for an additional 10 min. The chromatogram was collected in 20-s fractions, and flow rate was 1.5 mL/min throughout.

Gel Electrophoresis. Gel electrophoresis was performed according to the procedure of Jackowski and Kun (1983).

Preparation of 4-Diazoniobenzamide. Fifty-five milligrams of 4-aminobenzamide was dissolved in 20 mL of 0.15 M HCl and incubated for 20 min at 4 °C with 50 mg of $NaNO_2$ added as a solid and stirred. After incubation, the pH was set to 5.0–5.5 by addition of 3 M NaOH. Under these conditions 4-aminobenzamide was quantitatively converted to the diazonium form, as determined by UV spectroscopy (λ_{max} 270 nm for the diazonium form). At pH 5.0 the diazo compound is stable for at least 45 min at room temperature, at pH 7.4 its half-life is 45 min, and at pH 8.0 the half-life is 18 min, as determined by decay of absorbance at 270 nm.

Preparation of [3,5-$^3H_2$]-4-Diazoniobenzamide. This was carried out by first amidating commercial 3,5-diiodo-4-aminobenzoic acid, then exchanging tritium atoms for the iodine atoms on the aromatic ring to form [3,5-$^3H_2$]-4-aminobenzamide, and then diazotizing with $NaNO_2$. For the amidation step, a 50-mL solution containing 3,5-diiodo-4-aminobenzoic acid (50 mM), *N*-hydroxysuccinimide (100 mM), and dicyclohexylcarbodiimide (80 mM) was prepared in absolute dioxane and incubated for 2 h at 24 °C. Fifty milliliters of NH_4OH (2.9%) was added to the mixture and allowed to react overnight at ambient temperature. The mixture was filtered and the solid material washed with water, then with ether, and then extracted with dioxane (3 × 15 mL). The combined dioxane extract was rotary evaporated, and the residue was dissolved in 50% aqueous MeOH. The product was further purified by isocratic HPLC on an Ultrasphere ODS column eluted with 50% aqueous MeOH. Yield of product was 12.4% and its identity as 3,5-diiodo-4-aminobenzamide confirmed by its high-resolution mass spectrum, which displayed the expected molecular ion: *m/z* calculated for $C_7H_6I_2N_2O$, 387.8569; found, 387.8538 (deviation = 8.0 ppm). For the tritiation step approximately 15 silica–alumina catalyst pellets ($^1/_8$ in.) were impregnated with a total of 300 μL of a methanolic solution containing 2 mg of the above product, and after the pellets were dried in vacuo, they were subjected to microwave discharge in an atmosphere of tritium gas (Cao & Peng, 1983; Gordon et al., 1982; Peng et al., 1982). The product was then extracted from the pellets with MeOH, applied onto a preparative silica gel TLC plate, and developed with ethyl acetate. The position of the [3,5-$^3H_2$]-4-aminobenzamide was identified by nonradioactive markers, and the compound was eluted from the silica gel with MeOH and purified further by HPLC on the Ultrasphere ODS column with isocratic elution (25% aqueous MeOH). The specific radioactivity of the compound was 14.4 Ci/mmol. For the diazotization, the [3,5-$^3H_2$]-4-aminobenzamide prepared above was combined with nonradioactive 4-aminobenzamide to give a desired specific radioactivity and the mixture diazotized with $NaNO_2$ as described above for the preparation of 4-diazoniobenzamide.

Preparation of [3,5-$^3H_2$]-4-Diazoniobenzoic Acid. This was carried out exactly as described above for tritiated 4-aminobenzamide, except commercial 3,5-diiodo-4-aminobenzoic acid was not amidated but was directly subjected to tritiation on catalytic pellets to give [3,5-$^3H_2$]-4-aminobenzoic acid. After this compound was combined with nonradioactive 4-aminobenzoic acid as carrier, the material was diazotized with $NaNO_2$.

Preparation of the Chemical Adduct of 4-Diazoniobenzamide with dGMP. In 50 mL of 0.15 M HCl, 4-aminobenzamide (408 mg, 3.0 mmol) was diazotized with $NaNO_2$ (345 mg, 5.0 mmol) at 4 °C. Some of resultant 4-diazoniobenzamide salt precipitated, after 30 min the pH of the stirred mixture was adjusted to 6.8 with 2 M sodium hydroxide, and then dGMP (disodium salt hydrate, 250 mg, 0.57 mmol) was added. After the mixture was stirred for 1 h at 4 °C, it was allowed to stand at 24 °C for 14 h and filtered, and the red-orange filtrate stripped of water on a rotary evaporator. Analysis of a microsample of the residue on a silica gel TLC plate showed the presence of a product, distinct from starting materials and self-reaction products of the diazonium reagent, which was mobilized on silica gel by MeOH/$CHCl_3$ (80/20 v/v). The dried residue from the above rotary evaporation was dissolved in $CHCl_3$ and applied to a silica gel column (12 in. × 2 in.), which was then developed by successive addition of 100-mL portions of 0/100, 10/90, 20/80, 40/60, 60/40, 80/20, and 100/0 MeOH/$CHCl_3$ (v/v). The product eluted in the last three of these 100-mL fractions, which were pooled and concentrated to a volume of 10 mL (rotary evaporator), and this solution was subsequently chromatographed, portionwise in a series of injections, on the reversed-phase HPLC column with the elution gradient described under HPLC (see above). The product, which constituted the major peak, was collected, pooled, and desalted from the HPLC buffer by adsorption on Waters reversed-phase Sep-Pak cartridges. After being washed with water, the orange product was desorbed by acetonitrile/H_2O (50/50) and rotary evaporated to dryness, yielding 0.0132 g (4.3% yield) of the adduct as the monopotassium salt: UV λ_{max} 370 nm (ϵ 4.62 × 10^3) and λ_{max} 249 nm (ϵ 5.37 × 10^3) in water. The product was not recrystallized but was analyzed directly by NMR and mass spectrometry.

Protein concentrations were determined according to the method of Lowry et al. (1951).

Isolation of Rat Liver Nuclei and Assay of Poly(ADP-ribose) Polymerase Activity Therein. Rat liver nuclei were isolated according to Hakam et al. (1984) and Hakam and Kun (1985). The nuclear suspension (equivalent to 25–75 μg of DNA) was assayed for poly(ADP-ribose) polymerase enzyme activity with 9 μM [^{14}C]NAD^+ (adenine labeled, 290

Table I: Protective Effect of Benzamide against Inhibition of Poly(ADP-ribose) Polymerase by 1.6 mM 4-Diazoniobenzamide in Liver Nuclei and Ineffectivity of 4-Diazoniobenzoic Acid[a]

experiment	4-diazonio-benzoic acid, 1.6 mM	4-diazonio-benzamide, 1.6 mM	added benzamide concn (mM)	ADP-ribose products in nuclei (nmol of ADP-ribose/ mg of DNA formed in 1 min)[b]
1	-	-	-	1.3–1.6
2	-	-	7.7	1.2–1.5
3	-	+	-	0.2–0.4
4	-	+	2.3	0.6–0.8
5	-	+	7.7	1.2–1.5
6	+	-	-	1.2–1.4

[a] Suspensions of rat liver nuclei (6 mg of protein and 1.5 mg of DNA per 250 μL) were incubated with additions as indicated. Nuclei were preincubated at 4 °C for 10 min with benzamide and/or 4-diazoniobenzamide or 4-diazoniobenzoic acid, which were subsequently removed by two centrifugal washings (500*g* for 10 min). In all cases the enzymatic activity in the reisolated nuclei was assayed as described under Experimental Procedures. [b] The results are the ranges of three independent experiments.

mCi/mmol) as substrate, in 150 mM Tris-HCl (pH 8.0) containing 10 mM $MgCl_2$ and 1 mM dithiothreitol in a final volume of 50 μL. When mono-ADP-ribosylation was determined, the concentration of NAD^+ was 100 nM (Bauer et al., 1986).

Isolation of Calf Thymus Poly(ADP-ribose) Polymerase and Coenzymic DNA. Calf thymus poly(ADP-ribose) polymerase was purified to homogeneity, and coenzymic DNA was separated from the enzyme by hydroxylapatite chromatography according to the method of Yoshihara et al. (1978). Mono-ADP-ribosylation and poly-ADP-ribosylation were assayed according to Kirsten et al. (1985).

Results

Inhibition of the particulate matter bound poly(ADP-ribose) polymerase by 4-diazoniobenzamide was determined in rat liver nuclei. Liver nuclei were suitable for the determination of the reversibility of inhibition by benzamides. As demonstrated in Table I, experiment 2, the inhibitory effect of 7.7 mM benzamide, which was nearly 100% (not shown), was completely reversed by the reisolation of nuclei involving two centrifugal washings. Prior to diazotization 4-aminobenzamide is also a reversible inhibitor with a K_i = 75 μM competitive toward NAD^+. However, incubation of nuclei with 1.6 mM 4-diazoniobenzamide in an ice-water bath at pH 7.4 for 10 min followed by reisolation of nuclei (Table I) resulted in 86% inhibition of poly(ADP-ribose) polymerase activity (experiment 3, Table I), which was not reversed by centrifugal washing of nuclei. Since 4-diazoniobenzoic acid under identical conditions had no significant inhibitory effects (experiment 6, Table I), it was evident that the $-C(O)NH_2$ moiety of benzamide was required for enzyme inhibition by 4-diazoniobenzamide and the reaction of the $-N_2^+$ moiety with a component of the nuclear system was by itself not inhibitory. This assumption was directly tested (experiments 4 and 5, Table I) by the simultaneous incubation of nuclei with 1.6 mM 4-diazoniobenzamide and 2.3 and 7.7 mM benzamide, respectively. As assayed after reisolation of nuclei, the simultaneous presence of the reversible inhibitor, benzamide, with 4-diazoniobenzamide significantly protected the poly(ADP-ribose) polymerase system against the apparently irreversible inhibition by 4-diazoniobenzamide.

Results summarized in Table I suggest that the $-N_2^+$ moiety of 4-diazoniobenzamide may react covalently with a component of the poly(ADP-ribose) polymerase system, rendering the inhibitory effect of the $-C(O)NH_2$ part of benzamide irreversible to centrifugal washing. The rate of covalent binding of 4-diazoniobenzamide to a macromolecular component of the polymerase system would then be expected to be limiting in the ensuing enzyme inhibition. When the reaction of 4-diazoniobenzamide with nuclei was quenched by

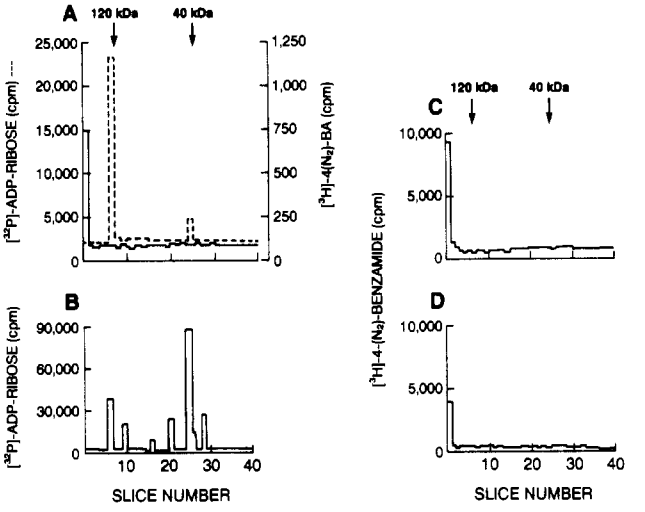

FIGURE 1: Labeling of protein and DNA components in a reconstituted poly(ADP-ribose) polymerase system and in liver nuclei, as determined with gel electrophoresis. (A) Purified poly(ADP-ribose) polymerase (5 μg), coenzymic DNA (0.2 μg), and total calf thymus histones (10 μg/mL) were incubated in a total volume of 50 μL of incubation buffer (Kirsten et al., 1985) with either 100 nM ^{32}P-labeled NAD^+ (1.1 Ci/μmol) for 1 min at 25 °C (---) or 0.6 mM [3,5-$^3H_2$]-4-diazoniobenzamide (5.6 μCi) in an ice–water bath for 30 min with the reaction terminated by addition of 50 μL of gel electrophoresis buffer containing 1 mg/mL (chloroacetyl)tyrosine (—). (B) Rat liver nuclei were labeled with [^{32}P]NAD^+ and [3,5-$^3H_2$]-4-diazoniobenzamide as described for (A), followed by gel electrophoretic separation of DNA and proteins. No ^3H was detected in gel slices, indicating only ADP-ribose labeling. (C) A suspension (10 μL) of liver nuclei (2 mg/mL protein) was allowed to react with [3,5-$^3H_2$]-4-diazoniobenzamide (5.1 μCi, 0.1 mM) at 4 °C for 30 min, and the reaction was terminated as described for (A), followed by gel electrophoresis (Jackowski & Kun, 1983). (D) Electrophoretogram illustrating the decay of DNA-bound [3,5-$^3H_2$]-4-diazoniobenzamide following digestion with 10 μg of DNase I for 30 min at 25 °C. The system was the same as for (C). Digestion with proteinase K had no effect. [^3H]-4(N_2)-BA denotes [3,5-$^3H_2$]-4-diazoniobenzamide.

an excess of (chloroacetyl)tyrosine (see legend of Figure 1) after various times of incubation with 4-diazoniobenzamide (from 5 to 20 min), at various concentrations of the inhibitor (between 0.25 and 2.5 mM), the enzymatic activity of the reisolated nuclei decayed with first-order kinetics and k_I was linear with the concentration of the inhibitor between 0.25 and 4 mM.

The macromolecular target of covalent binding of 4-diazoniobenzamide was identified as DNA by double-label experiments with [3,5-$^3H_2$]-4-diazoniobenzamide and [^{32}P]-NAD^+, respectively. The macromolecular products of an incubation system, composed of the purified enzyme, coenzymic DNA, [3,5-$^3H_2$]-4-diazoniobenzamide, [^{32}P]NAD^+, and a mixture of histones (see legend of Figure 1A), were separated in a gel (Jackowski & Kun, 1983) which retained DNA at

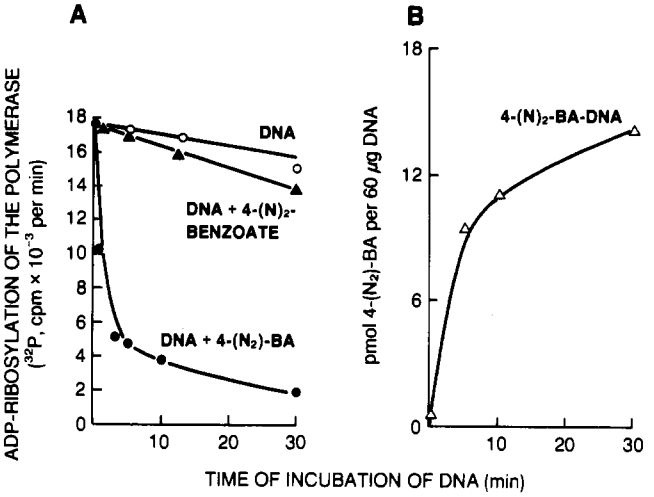

FIGURE 2: Effects of pretreatment of coenzymic DNA with 4-diazoniobenzamide or 4-diazoniobenzoic acid. (A) Rates of ADP-ribosylation were determined in the presence of coenzymic DNA that had been either not treated (○) or treated with 1 mM 4-diazoniobenzoic acid (▲) as controls. In the third experiment (●) the coenzymic DNA was treated with 1 mM 4-diazoniobenzamide. After exposure to diazo compounds at 4 °C, coenzymic DNA was extensively dialyzed (6 × 4 L of H_2O) and reprecipitated by EtOH (4 °C) to remove traces of decomposition products prior to enzymatic assays. Enzymatic assays were performed as described under Experimental Procedures. (B) After exposure of coenzymic DNA to 1 mM [3,5-$^3H_2$]-4-diazoniobenzamide, binding of the diazo compound was determined by acid precipitation and scintillation counting. Identical binding of [3,5-$^3H_2$]-4-diazoniobenzoic acid was also observed (not shown).

the origin and separated the enzyme (120-kDa component) and a trace amount of histone-like proteins (40 kDa, Figure 1A). As the NAD^+ concentration was 100 nM, only mono-ADP-ribosylation of the enzyme protein occurred (Bauer et al., 1986), avoiding artifacts of gel electrophoresis introduced by covalently bound poly(ADP-ribose). Similar results were obtained with isolated liver nuclei, except a number of mono-ADP-ribosylated proteins were isolated besides the polymerase enzyme by gel electrophoresis (Figure 1B). The only macromolecular component that was labeled with [3,5-$^3H_2$]-4-diazoniobenzamide was DNA (Figure 1C). Subsequent digestion of nuclei with DNAse I (20 μg/50 μL for 30 min at 25 °C) significantly reduced the ^3H label in the DNA region of the gel. Partial inaccessibility of nuclear DNA to external DNase I may explain the results (Figure 1D).

Experiments depicted in Figure 2 illustrate that incubation of coenzymic DNA with 1.6 mM 4-diazoniobenzamide, followed by reisolation of the modified DNA by EtOH precipitation and removal of degradation products, yielded a DNA that inhibited the purified enzyme as a function of incubation time with 4-diazoniobenzamide (descending curve in Figure 2A). Covalent binding of [3,5-$^3H_2$]-4-diazoniobenzamide or [3,5-$^3H_2$]-4-diazoniobenzoic acid (not shown) yielded kinetics of binding as shown in the curve of Figure 2B. Only the DNA adduct of 4-diazoniobenzamide was inhibitory, identifying the benzamide moiety as the inhibitory species. It is known that the catalytic coenzymatic function of coenzymic DNA can be, at least in part, replaced by synthetic oligodeoxyribonucleotides (Berger & Petzold, 1985). However, we find that the association of synthetic oligodeoxyribonucleotides to the polymerase protein is significantly weaker than that of macromolecular coenzymic DNA. For example, k_D for DNA (molecular mass 1.3×10^6 Da) is 1×10^{-9}, whereas for $(dA\text{-}dT)_8$ k_D is between 4×10^{-7} and 7×10^{-7} (A. Hakam, J. McLick, K. Buki, and E. Kun, unpublished results). Therefore, the inhibition of poly(ADP-ribose) polymerase by 4-diazoniobenzamide, which depends on tightly binding macromolecular coenzymic DNA, would not be reproduced by weakly binding synthetic DNA analogues.

The mechanism of covalent modification of DNA by 4-diazoniobenzamide was determined in two stages: first, dGMP was identified as the acceptor (Figure 3), and then the chemical structure of the dGMP adduct was clarified (Figure 4). The chemical degradation of 4-diazoniobenzamide is shown in Figure 3, chromatogram 1, benzamide being the main product. When nuclei were digested with nucleases (see legend of Figure 3) following incubation with [3,5-$^3H_2$]-4-diazoniobenzamide, and deoxyribonucleotide monophosphates were located in the chromatographic elution, a new [3,5-$^3H_2$]-4-diazoniobenzamide-labeled substance appeared (chromatogram 2, Figure 3). The same product was obtained when, instead of nuclei, coenzymic DNA was treated with [3,5-$^3H_2$]-4-diazoniobenzamide (chromatogram 3, Figure 3). Incubation of dGMP with [3,5-$^3H_2$]-4-diazoniobenzamide yielded a single product (chromatogram 4, Figure 3), which was further identified as a 1:1 adduct of dGMP and 4-diazoniobenzamide by double labeling (chromatogram 5, Figure 3).

Preparative-scale synthesis of the dGMP adduct for structure elucidation was achieved by reacting 0.57 mmol of dGMP with an excess of 4-diazoniobenzamide in aqueous solution at pH 6.8. Purification by silica gel column chromatography and reversed-phase HPLC gave a product that had the same elution position as the adduct isolated from the coenzymic DNA (chromatogram 3, Figure 3) as well as the doubly labeled adduct (chromatogram 5, Figure 3) with a yield of 13.2 mg of adduct (4.3%). The molecular structure deduced for the adduct, N^2-[(4-carbamoylphenyl)azo]-2′-deoxyguanosine 5′-monophosphate monopotassium salt, is depicted in Figure 4 together with spectra obtained by ^1H NMR and liquid matrix secondary ionization mass spectrometry (LSIMS) (Falick et al., 1986). That the adduct was formed by substitution of a 4-diazoniobenzamide group for a hydrogen on the dGMP molecule (monopotassium), with a resultant molecular weight of 532 ($C_{17}H_{18}N_8O_8KP$), is demonstrated in the mass spectrum by (a) the molecular ion peak at m/z 531 corresponding to the adduct molecule minus a proton [$(M - H)^-$], (b) m/z 493 corresponding to the adduct minus a potassium ion [$(M - K)^-$], (c) m/z 384 corresponding to the adduct minus the 4-diazoniobenzamide ion [$(M - 148)^-$], and (d) m/z 346 corresponding to the adduct minus the potassium ion and the 4-diazoniobenzamide group less one hydrogen [$(M - K - 137)^-$]. That the 4-diazoniobenzamide group is specifically located on the amino group (N^2) of the guanine moiety is indicated in the ^1H NMR spectrum by (a) the presence of only a single guanine N^2-H hydrogen (peak area equal to that of the ribose $H_{1'}$ hydrogen) and (b) the washout of the lone N^2-H hydrogen in deuterium oxide. A previous example of adduct formation (with 6-methylbenzo[α]pyrene) on the amino group of guanine with concomitant loss of a hydrogen has been reported by Rogan et al. (1983).

The structure of the adduct has the Z (or anti) geometry assigned about the nitrogen–nitrogen double bond, but the geometry could also be E (or syn), and additionally, the triazene group may undergo tautomerization [cf. Wulfman (1978)].

If the adduct of 4-diazoniobenzamide to dGMP is the molecular species that represents the covalently bound 4-diazoniobenzamide to DNA, certain similarities would be expected between the inhibition of poly(ADP-ribose) polymerase by benzamide (Purnell & Whish, 1980) and by the

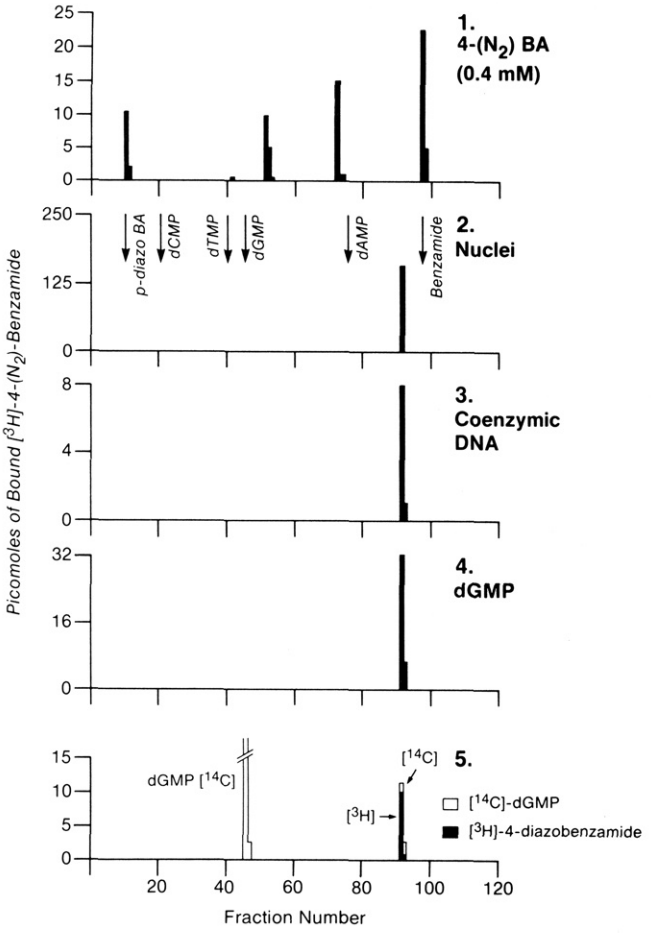

FIGURE 3: Identification of dGMP as target of 4-diazoniobenzamide. Samples were incubated with [3,5-$^3H_2$]-4-diazoniobenzamide in 10 mM Tris-HCl (pH 7.4), 10 mM $MgCl_2$, 1 mM dithiothreitol, and 0.25 M sucrose, at 4 °C for 60 min. Incubates were then appropriately treated (see below) for HPLC analysis as described under Experimental Procedures, and 20-s fractions were collected and counted by liquid scintillation. (1) HPLC pattern of degradation products of 4-diazoniobenzamide. [3,5-$^3H_2$]-4-Diazoniobenzamide (5 μCi, 0.9 mM) in a total volume of 25 μL of buffer (see above) was incubated, and 10 μL was directly injected into the HPLC. Note the major degradation product is benzamide. (2) Covalent binding of 4-diazoniobenzamide to DNA in nuclei. [3,5-$^3H_2$]-4-diazoniobenzamide (50 μCi, 1 mM) was added to rat liver nuclei (containing 5.5 mg of DNA) in a total volume of 2.5 mL of incubation buffer. At the end of incubation the mixture was diluted towfold with additional buffer containing (chloroacetyl)tyrosine (1 mg/mL) as quenching agent. The incubate was then deproteinized by phenol extraction (twice) followed by precipitation of nucleic acids by addition of 2 volumes of EtOH. To the precipitate was added 3 M NH_4OH (37 °C for 6 h) to hydrolyze traces of RNA. After freeze-drying, the sample was redissolved in 2 mL of 50 mM Tris-HCl (pH 7.4) and 20 mM NaCl, followed by another precipitation with EtOH. The DNA was dissolved in Tris-HCl (pH 8.0, 100 mM) containing 100 mM $MgCl_2$ and 100 mM $CaCl_2$ and then completely digested with DNAse I (1 mg/mL) plus Bal-31 nuclease (25 units per test) for 6 h at 37 °C. The products of digestion were directly injected into the HPLC system. The elution positions of 2′-deoxynucleotide, 4-diazoniobenzamide, and benzamide standards are indicated by arrows. (3) Covalent binding of 4-diazoniobenzamide to coenzymic DNA. [3,5-$^3H_2$]-4-diazoniobenzamide (5 μCi, 0.4 mM) was added to 13 μg of coenzymic DNA in a total volume of 50 μL of incubation buffer. After incubation the sample was treated the same as that of (2) above. (4) Covalent binding of 4-diazoniobenzamide to dGMP. [3,5-$^3H_2$]-4-diazoniobenzamide (5 μCi, 0.9 mM) in a total volume of 50 μL of buffer was incubated with dGMP (10 mM) and directly analyzed by HPLC. Background radioactivity was subtracted from the chromatogram. Under identical conditions, dAMP, dTMP, and dCMP did not form any adduct. (5) Same as (4) except 160 μM [^{14}C]dGMP (500 μCi/mmol) was incubated with [3,5-$^3H_2$]-4-diazoniobenzamide and the doubly labeled product was isolated by HPLC.

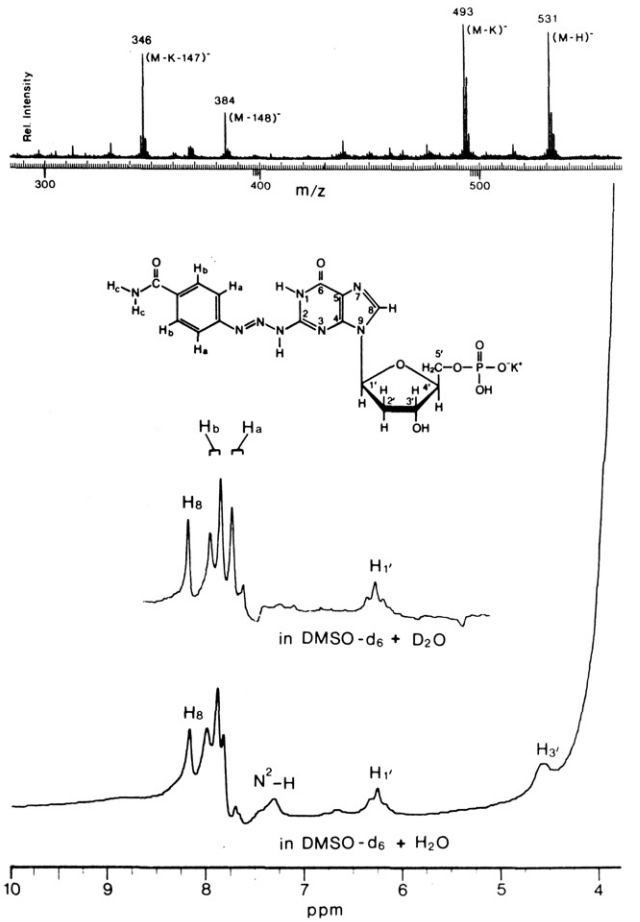

FIGURE 4: LSIMS and ^{1}H NMR spectra and structure of the synthetic adduct of 4-diazoniobenzamide with dGMP. The LSIMS spectrum was obtained from the adduct suspended in a glycerol matrix. No other major peaks were observed except those displayed. To obtain the NMR spectrum, it was necessary to add water to solubilize the adduct in Me_2SO-d_6 solvent; thus a large water resonance peak was incurred below 6 ppm, obscuring most of the ribose resonances except H_1' and H_3'. Chemical shifts are relative to internal tetramethylsilane standard. The spectrum obtained after equilibration with deuterium oxide is shown in the inset, whose vertical scale has been expanded by 40%. In deuterium oxide the N^2-H hydrogen is washed out, as are the benzamide amino hydrogens (H_c). In the nondeuterium spectrum these benzamide hydrogens overlap the aromatic ring hydrogens (H_a and H_b), in the region of 7.70–8.00 ppm, as in benzamide itself (Pouchert, 1983) and thus obscure each other. In the deuterium-equilibrated spectrum the resonance pattern of the ring hydrogens is disclosed, and as is typical for para-disubstituted benzenes, a pattern of two doublets occurs with the inner two peaks of much greater intensity than the outer two (Silverstein et al., 1981). Not shown is the guanine N^1-H resonance (broad adsorption around 11.59 ppm) which is known to occur at chemical shifts greater than 10 ppm (Shoup et al., 1966).

adduct. Whereas benzamide inhibited the enzyme by competing with NAD^+ (k_i = 2 μM), inhibition by the adduct was weaker (k_i = 150 μM) and noncompetitive with respect to NAD^+, indicating an altered binding of the adduct to the enzyme as compared to benzamide. Since the in vitro enzyme system contains excess coenzymic DNA, it would be anticipated that DNA could compete with the dGMP moiety of the adduct for binding sites on the enzyme that may be common for both DNA and dGMP, thus diminishing the enzyme inhibitory effect of the 4-diazoniobenzamide–dGMP adduct and also altering the type of inhibition. More detailed kinetic analyses are the subject of further work.

Discussion

The diazonium derivative of 4-aminobenzamide could

predictably react with tyrosine, histidine, or lysine residues of protein (Riordan & Valee, 1972), and isolation of these adducts should identify peptide structures presumably vicinal to the NAD^+-binding site of the polymerase protein. However, at low concentrations of 4-diazoniobenzamide (or of 4-diazoniobenzoic acid) not exceeding 2 mM, preferential reaction with dGMP of DNA occurs. In agreement with Riordan and Valee (1972), at 10–20 mM 4-diazoniobenzamide the protein components of isolated nuclei also started to become labeled with the tritiated reagents; thus specificity toward dGMP was lost. Therefore, our results are restricted to 1–2 mM 4-diazoniobenzamide. The structure assigned to the adduct, N^2-[(4-carbamoylphenyl)azo]-2′-deoxyguanosine 5′-monophosphate monopotassium salt, features a triazene grouping of nitrogen atoms, known to form from the reaction of arenediazonium ions with arylamines at neutral conditions (Wulfman, 1978). It is not clear why dAMP does not react with 4-diazoniobenzamide, since the reactivity of the NH_2 group in both deoxyribonucleotides should be similar. The calculated specific activity of DNA, representing moles of 4-diazoniobenzamide per micromole of base, was 200 pmol/μmol of base; i.e., 1 out of 5000 bases reacted with the diazonium salt. The coenzymic DNA strongly binds to the poly(ADP-ribose) polymerase protein (k_D = 1.0 nM, unpublished results); therefore, the inhibition of the enzyme at the $-C(O)NH_2$-binding site, imitating irreversible inactivation, can be explained by the tight DNA–protein association, keeping the benzamide moiety at the NAD^+ site of the enzyme, which is a unique feature of this inhibition mechanism. It also follows that the distance on the enzyme protein between the nicotinamide-binding site and the DNA-binding domain, which contains the covalently bound 4-diazoniobenzamide residue attached to DNA, is on the order of 8.4 Å, calculated from interatomic distances estimated for the triazene compound (Figure 4). Assuming DNA is in the B form, the covalent adduct linkage is probably in the minor groove of the DNA (Petrusek et al., 1981).

Whereas our results are consistent with the proposed mechanism that the $-C(O)NH_2$ group is responsible for enzyme inhibition by the 4-diazoniobenzamide–dGMP adduct, the specificity of dGMP as the target of 4-diazoniobenzamide in DNA is not readily explained, unless one assumes that the macromolecular structural contribution of coenzymic DNA (molecular mass 1.3×10^6) plays an important but as yet unknown function in directing 4-diazoniobenzamide toward dGMP.

Conformational changes in the enzyme protein are likely to modify the relatively small distance between the nicotinamide site and the DNA-binding domain, predicting allosteric mechanisms. There is some experimental evidence to support the participation of allosteric mechanisms. The large activation of automono-ADP-ribosylation of the polymerase protein in the presence of 12 nM nicotinic acid analogue of NAD^+ (with 25 nM NAD^+ as ADP-ribose donor) in difficult to explain without invoking unorthodox kinetic models (Bauer et al., 1986).

References

Bauer, P. I., Hakam, A., & Kun, E. (1986) *FEBS Lett. 195*, 331–338.

Benjamin, R. C., & Gill, D. M. (1980a) *J. Biol. Chem. 255*, 10493–10501.

Benjamin, R. C., & Gill, D. M. (1980b) *J. Biol. Chem. 255*, 10502–10508.

Berger, N. A., & Petzold, S. J. (1985) *Biochemistry 24*, 4352–4355.

Cao, C. Y., & Peng, C. T. (1983) *Trans. Am. Nucl. Soc. 45*, 18–19.

DeMurcia, G., Jongstra-Bilen, J., Ittel, M. E., Mandel, P., & Delain, E. (1983) *EMBO J. 2*, 543–548.

Falick, A. M., Wang, G. H., & Walls, F. C. (1986) *Anal. Chem. 58*, 1308–1311.

Gaal, J. C., & Pearson, C. K. (1985) *Biochem. J. 230*, 1–18.

Gordon, B. E., Peng, C. T., Erwin, W. R., & Lemmon, R. M. (1982) *Int. J. Appl. Radiat. Isot. 33*, 715–720.

Hakam, A., & Kun, E. (1985) *J. Chromatogr. 330*, 287–298.

Hakam, A., McLick, J., & Kun, E. (1984) *J. Chromatogr. 296*, 369–377.

Ittel, M. E., Jongstra-Bilen, J., Niedergang, C., Mandel, P., & Delain, E. (1985) in *ADP-Ribosylation of Proteins* (Althaus, F. R., Hilz, H., & Shall, S., Eds.) pp 60–68, Springer-Verlag, New York.

Jackowski, G., & Kun, E. (1983) *J. Biol. Chem. 258*, 12587–12593.

Kameshita, I., Matsuda, M., Nishikimi, M., Ushiro, H., & Shizuta, Y. (1986) *J. Biol. Chem. 261*, 3863–3868.

Kawaichi, M., Ueda, K., & Hayaishi, O. (1981) *J. Biol. Chem. 256*, 9483–9489.

Kirsten, E., Jackowski, G., McLick, J., Hakam, A., Decker, K., & Kun, E. (1985) *Exp. Cell. Res. 161*, 41–52.

Lowry, O. H., Rosebrough, N. J., Farr, A. L., & Randall, R. J. (1951) *J. Biol. Chem. 193*, 265–275.

Miller, E. G. (1975) *Biochim. Biophys. Acta 395*, 191–200.

Niedergang, C., Okazaki, H., & Mandel, P. (1979) *Eur. J. Biochem. 102*, 43–47.

Peng, C. T., Gordon, B. E., Erwin, W. R., & Lemmon, R. M. (1982) *Int. J. Appl. Radiat. Isot. 33*, 419–427.

Petrusek, R. L., Anderson, G. L., Garner, T. F., Fannin, Q. L., Kaplan, D. J., Zimmer, S. G., & Hurley, L. H. (1981) *Biochemistry 20*, 1111–1119.

Pouchert, C. J. (1983) *The Aldrich Library of NMR Spectra*, 2nd ed., Vol. 2, p 343D, Aldrich Chemical Co., Milwaukee.

Purnell, M. R., & Whish, W. J. D. (1980) *Biochem. J. 185*, 775–777.

Riordan, J. F., & Valee, B. L. (1972) *Methods Enzymol. 25B*, 521–531.

Rogan, E. G., Hakam, A., & Cavalieri, E. L. (1983) *Chem.-Biol. Interact. 47*, 111–122.

Shoup, R. R., Miles, H. T., & Becker, E. D. (1966) *Biochem. Biophys. Res. Commun. 23*, 194–201.

Silverstein, R. M., Bassler, G. C., & Morrill, T. C. (1981) *Spectrometric Identification of Organic Compounds*, 4th ed., pp 190–191, Wiley, New York.

Skidmore, C. J., Jones, J., Oxberry, J. M., Chaudun, E., & Counis, M.-F. (1985) in *ADP-Ribosylation of Proteins* (Althaus, F. R., Hilz, H., & Shall, S., Eds.) pp 116–123, Springer-Verlag, New York.

Ueda, K., & Hayaishi, O. (1985) *Annu. Rev. Biochem. 54*, 73–100.

Wulfman, D. S. (1978) in *The Chemistry of Diazonium and Diazo Groups, Part 1* (Patai, S., Ed.) pp 261–262, Wiley, New York.

Yoshihara, K., Hashida, T., Tanaka, Y., Ohgushi, H., & Kamiya, T. (1978) *J. Biol. Chem. 253*, 6459–6466.