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Use of Substituted (Benzylidineamino)guanidines in the Study of Guanidino Group Specific ADP-ribosyltransferase†

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ABSTRACT: A number of substituted (benzylidineamino)guanidines with different substitutents in the benzene nucleus are synthesized by coupling substituted benzaldehydes with aminoguanidine, and these compounds are tested as substrates for cholera toxin catalyzed ADP-ribosylation. A spectrophotometric assay method for the measurement of ADP-ribosyltransferase activity is developed, making use of the absorption characteristics of some of these compounds and the difference in the ionic character of the free compounds and the ADP-ribosylated products. The kinetic parameters for the ADP-ribosylation of these compounds are evaluated. A correlation between log k_{cat} or log (k_{cat}/K_m) and the Hammett substituent constant σ is observed. This correlation suggests the importance of substrate electronic effects on the enzymatic reaction. The reactivity of these compounds as acceptors of ADP-ribosyl groups in the reaction catalyzed by cholera toxin increases with increasing electron-donating power of the substituents in the benzene function. The effect is primarily on the catalytic rate constant, k_{cat}, not on the binding constant, K_m. The results are consistent with an S_N2 reaction mechanism in which the deprotonated guanidino group makes a nucleophilic attack on the C-1 carbon of the ribose moiety.

Cholera toxin (Moss & Vaughan, 1977; Moss & Richardson, 1978; Moss et al., 1976), *Escherichia coli* enterotoxin (Moss & Richardson, 1978; Moss et al., 1979), and ADP-ribosyltransferases from different animal sources (Moss & Vaughan, 1978; Beckner & Blecher, 1981; Moss & Stanley, 1981a,b; Richter et al., 1983; Tanigawa et al., 1984; Soman et al., 1984a) are capable of transferring an ADP-ribosyl moiety from NAD[1] to functional groups in cellular proteins and also to simple guanidino compounds. The site of self-ADP-ribosylation in cholera toxin and the cholera toxin catalyzed ADP-ribosylation in transducin has been established to be arginyl residues (Xia et al., 1984; Van Dop et al., 1984). Though the functional residues modified in other proteins by cholera toxin and other ADP-ribosyltransferases are not well established, enzymes that use guanidino compounds as substrates are presumed to be guanidino group specific. Because of the recent reports indicating the presence of the ADP-ribosyltransferase activity in different animal and plant tissues (Moss & Vaughan, 1978; Beckner & Blecher, 1981; Moss & Stanley, 1981a,b; Richter et al., 1983; Tanigawa et al., 1984; Soman et al., 1984a; Pope et al., 1985), there is growing interest in these reactions as a means of posttranslational covalent modification of cellular proteins. But very little information is available on the natural protein substrates for these enzymes, on the precise physiological role of these reactions, and also on the enzymology of the process. In the absence of well-defined natural substrates, much information may be derived from studies using model substrates. Earlier studies on cholera toxin reveal that introduction of negatively charged groups near the guanidino group is unfavorable for binding (Moss & Vaughan, 1977). Thus, arginine itself is a very poor substrate for ADP-ribosylation by cholera toxin, but blocking the carboxyl group as in L-arginine methyl ester or replacing the carboxyl group by a methyl group as in agamatine makes the derivatives much better substrates for cholera toxin and other ADP-ribosyltransferases (Moss & Vaughan, 1977; Moss et al., 1983). A recent report suggests that, in a series of guanidino compounds tested, the ADP-ribosyltransferase activity, as measured by the ability of the compounds to release nicotinamide, increases with increasing hydrophobicity of the compounds (Tait & Nassau, 1984). Thus, both electronic effects and hydrophobicity seem to influence the reactivity of the ADP-ribosyltransferases.

Recently, we have reported that the guanylhydrazones, [(*p*-nitrobenzylidine)amino]guanidine and methylglyoxal

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[1] Abbreviations: TEAA, triethylammonium acetate; BAG, (benzylidineamino)guanidine; DEA, diethylamino; DMA, dimethylamino; NBAG, [(*p*-nitrobenzylidine)amino]guanidine; HPLC, high-performance liquid chromatography; TCA, trichloroacetic acid; LAME, L-arginine methyl ester; NAD, nicotinamide adenine dinucleotide; DTE, dithioerythritol; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride.

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bis(guanylhydrazone), are good substrates for ADP-ribosyltransferases (Soman et al., 1983, 1984b). Both these compounds have low pK_a values for the guanidino group, and the former compound is also hydrophobic. Assay methods for the measurement of guanidino group specific ADP-ribosyltransferase activity has been reported with use of the former compound as a model substrate (Soman et al., 1983). The (benzylidineamino)guanidine has a guanidino group directly attached to a fully conjugated benzylidineamino group. Derivatives of the compound with differing substituents in the benzene nucleus would provide a very simple model system for structure–activity relationship studies for cholera toxin and other guanidino group specific ADP-ribosyltransferases. In this paper we report the synthesis, physical characterization, and use of different derivatives of (benzylidineamino)guanidine in the structure–activity relationship studies with cholera toxin. Also, a simple spectrophotometric assay method for the measurement of guanidino group specific ADP-ribosyltransferases is developed, making use of the distinct spectral characteristics of some derivatives of (benzylidineamino)-guanidine and the charge difference between the free compounds and ADP-ribosylated product.

Materials and Methods

Cholera toxin, cholera toxin A subunit, NAD, and DTE were obtained from Sigma. The substituted benzaldehydes and aminoguanidine bicarbonate were obtained from Aldrich; Dowex 50W-X4 was obtained from Bio-Rad.

Elemental analysis was performed by Galbraith Laboratories. Spectral studies and absorption measurements were carried out in a Beckman Model DU-7 spectrophotometer fitted with a Beckman kinetics temperature regulator.

High-performance liquid chromatographic analyses were done on a Rainin Model Rabbit HP/HPX pump device attached to an Apple IIe PC controller and a Beckman Model 165 variable-wavelength detector or on a Beckman Model 100A/110A pump system attached to a Beckman Model 420 controller and a Hitachi Model 100-40 spectrophotometer detector. The column used was a 25 cm × 4.6 mm Microsorb C_{18} from Rainin. Chromatography was performed under isocratic conditions for the assay of ADP-ribosyltransferase activity by using different guanylhydrazones. The buffer system used was 0.2 M TEAA and 0.1 M perchlorate (pH 6.0). The methanol composition was optimized for each compound. The absorption of the eluent was recorded at the wavelengths corresponding to maximum absorption in acid pH, except for DEA-BAG and DMA-BAG, where absorption was recorded at 355 and 345 nm, respectively. The other details of the assay procedure are similar to those reported earlier for [(*p*-nitrobenzylidine)amino]guanidine (Soman et al., 1983).

The Dowex 50W-X4 resin used in this study was pretreated with acid and alkali before use as described below. The resin was first soaked in 1 N HCl for 15 min and washed with several liters of distilled water. The acid-washed resin was then soaked in 1 N NaOH for 30 min, filtered, and washed with several liters of distilled water.

Spectrophotometric assay of ADP-ribosyltransferase activity of cholera toxin was done by using the *p*-diethylamino or *p*-dimethylamino derivatives of (benzylidineamino)guanidine. The routine reaction system (125 μL) contained 10 mM NAD, 20 mM DTE, 50 mM Tris-HCl (pH 7.5), 2.0 mM DEA-BAG or 2.0 mM DMA-BAG, and 10–25 μg/mL cholera toxin A subunit. Control samples are set without cholera toxin. After a 10–15-min incubation at 30 °C, 100 μL of the reaction mixture is transferred to 150–200 mg of Dowex 50W-X4 resin suspended in 2.0 mL of 20 mM phosphate (pH 7.4). The mixture is shaken well for 0.5 min, and the resin is allowed to settle. The absorption of the clear supernatant is measured at 355 nm for DEA-BAG or 345 nm for DMA-BAG.

ADP-ribosyltransferase activity with other BAG derivatives was also measured spectrophotometrically as described above with minor modifications. After the reaction, the samples (100 μL) are transferred to 150–200 mg of resin suspended in water and mixed well. The resin is allowed to settle. A 100-μL aliquot of 1 N NaOH is then added to 900 μL of the clear supernatant, and the absorption is recorded at the wavelengths corresponding to maximum absorption in alkaline pH indicated in Table I. The absorption values are converted to amount of product formed by using the known values of molar absorptivity of the respective BAG derivatives under the assay conditions.

Synthesis of Substituted (Benzylidineamino)guanidines. The (benzylidineamino)guanidine derivatives are synthesized by coupling aminoguanidine with the respective benzaldehyde derivatives according to the procedure reported earlier (Soman et al., 1983, 1984b). Because the reactions were incomplete in many cases and because the products do not crystallize easily, crystals of the free bases were obtained as follows: the reaction system after the coupling reaction (Baiocchi et al., 1963) is completely solubilized by adding ethanol. The clear solution is then stirred with 20–25 g of pretreated Dowex 50W-X4 resin. The supernatant is filtered off and the resin washed with 500 mL of 50% ethanol. The unreacted aldehydes are washed off while the product is adsorbed on the resin. The product is then eluted with 0.1 N NaOH in 50% ethanol. The resin is washed 3–4 times with 0.1 N NaOH in 50% ethanol, and the combined filtrate is neutralized with HCl and rotary-evaporated. The residue is dissolved in ethanol. The undissolved salt is removed by filtration, the ethanol is evaporated, and the process is repeated an additional 2 times. The residue is dissolved in water, and the pH is brought to 11–12 by addition of NaOH. The free bases of the compounds are extracted with ether 3–4 times. The combined ether layer is collected and evaporated, and the residue is dissolved in hot water (ethanol is added if necessary to just dissolve the residue completely). Crystals of the free bases are formed on cooling. The crystals are redissolved in hot water and recrystallized.

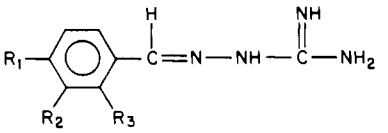
Kinetic parameters were obtained from a weighted least-squares fit employing the OMNITAB program of Siano et al. (1975). Regression analysis was performed by utilizing programs of the Statistical Analysis Package (SAS Institute, Cary, NC).

Physical Characterization of the Compounds. The pK_a values of the (benzylidineamino)guanidine derivatives were determined by spectrophotometric titration. Solutions of the compounds (0.05 mM) were prepared in buffers at different pHs and kept at room temperature for 30 min, and the absorptions were recorded at dual wavelengths corresponding to the absorption maxima in acid and alkali. The pK_a values were evaluated from the titration curves. Hydrophobicities of the different compounds were calculated from measuring the distribution of the compounds in water–octanol systems (Fujita et al., 1964).

Results

Physical Characteristics of the Guanylhydrazones. The compounds reported in this paper are obtained in crystalline form as free bases. The melting points, absorption characteritics, pK_a values, and hydrophobicities of the different compounds are tabulated in Table I. Figure 1 shows the dependence of the measured pK_a values on the substituent constant σ^+ (Hansch & Leo, 1979; Exner, 1978); there is good

Table I: Physical Characteristics of Substituted (Benzylidineamino)guanidines



compd no.	substituent R_1	R_2	R_3	mp (°C)	spectral characteristics: λ_{max} in 0.1 N HCl	λ_{max} in 0.1 N NaOH	log P_X[a]	pK_a
1	H	H	H	179–180	280	310	0.0188	8.7
2	NO_2	H	H	202–204	315	375	0.8650	8.2
3	Cl	H	H	199–200	284.5	314.5	0.9635	8.5
4[b]	Br	H	H	209–211	285.5	314.5	1.3022	8.5
5[b]	OCH_3	H	H	184–186	288.5	314.5	0.2909	9.2
6[b]	OC_7H_5	H	H	156–158	289.0	314.5	0.2419	9.3
7	$OC(CH_3)_3$	H	H	186–187	289.0	315	1.3801	9.25
8	phenoxy	H	H	174–176	288.5	314.5	1.8650	8.7
9[b]	phenyl	H	H	226–229	305	327	1.4892	8.75
10[b]	$N(CH_3)_2$	H	H	146–148	280	345	0.2800	9.8[c] (4.2)
11	$N(C_2H_5)_2$	H	H	141–142	280	355	0.5600	10.4[c] (5.1)
12[b]	H	NO_2	H	209–210	272.5	304	0.0123	8.35
13[b]	Cl	NO_2	H	201–202	282	317	0.5284	8.30
14	H	H	NO_2	182–184	259	286	0.5319	7.95

[a] Hydrophobicities of the respective compounds. [b] Elemental analysis of representative compounds is as follows: Anal. Calcd for **4**: C, 39.82; N, 23.23; H, 3.73; Br, 33.19. Found: C, 40.1; N, 23.08; H, 3.64; Br, 32.35. Anal. Calcd for **5**: C, 56.25, N, 29.16; H, 6.25. Found: C, 56.23; N, 29.42; H, 6.11. Anal. Calcd for **6**: C, 58.25; N, 27.18, H, 6.79. Found: C, 58.4; N, 26.93; H, 6.74. Anal. Calcd for **9**: C, 70.06; N, 23.53; H, 5.9. Found: C, 70.13; N, 23.8; H, 6.08. Anal. Calcd for **10**: C, 58.53; N, 34.14; H, 7.31. Found: C, 58.38; N, 33.89; H, 7.30. Anal. Calcd for **12**: C, 46.37; N, 33.82; H, 4.35. Found: C, 46.66; N, 34.00; H, 4.30. Anal. Calcd for **13**: C, 39.75; N, 28.9; H, 3.31. Found: C, 39.62; N, 28.95; H, 3.42. [c] Values calculated from Figure 1; the values in parentheses correspond to the experimentally determined pK_a values of the DMA and DEA groups.

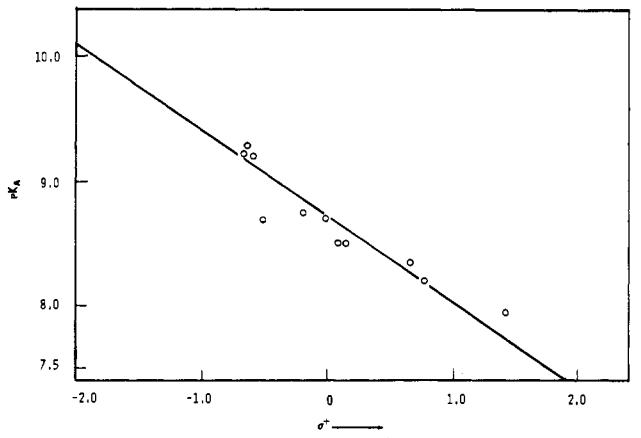


FIGURE 1: Dependence of pK_a values of substituted BAG compounds on substituent constants. σ^+ values were obtained from Hansch and Leo (1979) and Exner (1978). pK_a values were determined as described under Materials and Methods.

correlation of the experimental values with σ^+ values. The correlation of pK_a values with the simple Hammett substituent constants, σ, was poor. The titration of the DEA-BAG and DMA-BAG showed only one spectral shift in the entire pH range of 1–13. The pK_a evaluated corresponds to the titration of the dimethylamino or diethylamino group, inasmuchas the absorption characteristics and the pK_a values are comparable to that of the corresponding aldehydes. Though there is a shoulder in the absorption spectrum, around 315–320 nm in the neutral to alkaline pH range, attempts to evaluate the pK_a value of the guanidino group by following the spectral shift in this wavelength range were not successful. The values given in the table are those calculated from the Hammett plot (Figure 1).

Spectrophotometric Assay of Mono-ADP-ribosyltransferase Activity. All the substituted (benzylidineamino)guanidines are adsorbed in Dowex 50W-X4 resin in the neutral pH range. Figure 2 shows the adsorption of free NBAG and the lack of

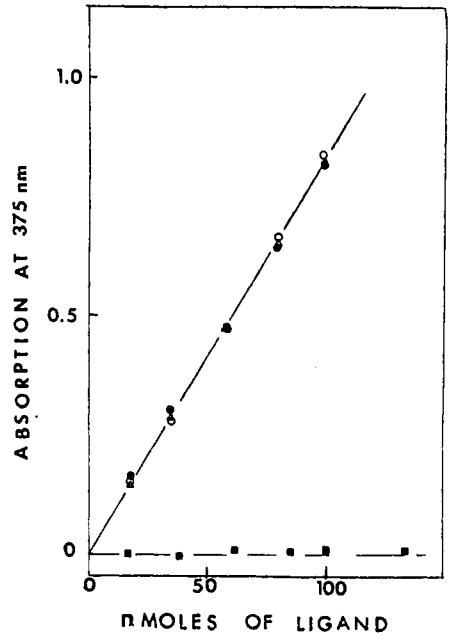


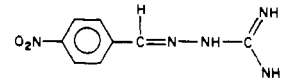
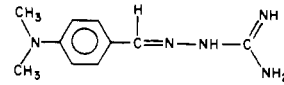
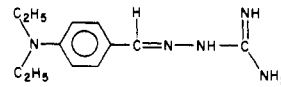
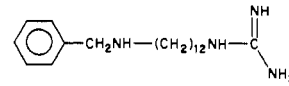
FIGURE 2: Adsorption characteristics of NBAG and ADP-ribosyl-NBAG on Dowex 50W-X4 resin. Different amounts of NBAG (■) and ADP-ribosylated NBAG (●) and 500 nmol of NBAG plus different amounts of ADP-ribosylated NBAG (△) were added to 150–200 mg of Dowex 50W-X4 resin suspended in 2.0 mL of distilled water. The samples are shaken well, and the resin is allowed to settle. A 900-μL aliquot of the clear supernatant is transferred to tubes containing 100 μL of 1 N NaOH. The solutions are mixed and the absorptions recorded at 375 nm. (○) Different amounts of ADP-ribosylated NBAG are added to 2.0 mL of distilled water and mixed, 900 μL is transferred to 100 μL of 1 N NaOH, and the absorption is recorded at 375 nm.

adsorption of ADP-ribosylated product on the Dowex resin. There is practically no adsorption of the ADP-ribosyl-NBAG both in the presence and in the absence of added NBAG, while there was quantitative adsorption of free NBAG both in the presence and in the absence of added ADP-ribosyl-NBAG.

These results are expected inasmuchas ADP-ribosylation introduces additional negative charges in the molecule, which can alter the adsorption characteristics of the compound in ion-exchange resins. Because some of the guanylhydrazones reported in this paper have strong absorption bands in wavelength regions far removed from that of the other components of the ADP-ribosyltransferase assay system, we have examined the applicability of this principle in developing a sensitive spectrophotometric assay for guanidino group specific ADP-ribosyltransferase. Studies with NBAG and cholera toxin showed that quantification of the ADP-ribosyltransferase activity of cholera toxin is possible by simply treating the reaction systems at different intervals with the resin suspended in water and reading the absorption of the supernatant at 375 nm after making the supernatant alkaline by adding NaOH. Our initial studies with the substituted (benzylidineamino)-guanidines revealed that the turnover rate with NBAG is much lower than that with many other compounds. Therefore, we examined other compounds as model substrates for a suitable assay. Two of the compounds, DEA-BAG and DMA-BAG, were particularly interesting for the following reasons: (1) These compounds absorb strongly at 355 or 345 nm, respectively, in the pH range 5 and above. The other components of the reaction system have no absorption at these wavelengths. (2) The turnover rate with these compounds is at least 1 order of magnitude higher than that observed with NBAG. (3) These two compounds are quantitatively adsorbed in the resin suspended in 20 mM phosphate buffer at pH 7.4. Therefore, a quantification of the reaction will be possible by directly measuring the absorption of the supernatant after treating with the resin provided that the products are not adsorbed in the resin. Figure 3A shows a linear time-dependent increase in absorption with this procedure. Also included in the figure is the time-dependent increase in the amount of products formed as estimated by integrating the areas of the product peaks in HPLC. The pattern is very similar to that reported earlier with the NBAG. Similar elution profiles were observed with all other compounds. In all cases, dual-product peaks are observed, and we presume that they probably are interconvertible anomeric forms of the ADP-ribosyl derivatives of guanylhydrazones for reasons discussed earlier (Soman et al., 1983). The amount of product formed is obtained by integrating the sum of the areas of the product peaks or by measuring the decrease in the area of the reactant peaks. The results in both cases were comparable. Quantification of the amount of product formed from the absorption measurements after resin treatment was done by presuming that the molar absorption of the ADP-ribosyl product is same as that of the free guanylhydrazone at 355 nm. The result so obtained was comparable to the result obtained from HPLC (Figure 3B). There was linear dependence of activity on cholera toxin concentration over a concentration range of 5–50 μg/mL cholera toxin A subunit (results not shown). In all cases, the results obtained by the spectrophotometric and HPLC methods are in reasonably good agreement and compatible with the characteristics expected for a typical enzymatic reaction. Thus, we suggest that a quantification of the ADP-ribosyltransferase activity of cholera toxin is possible by measuring the absorption with Dowex 50W-X4 resin as described in the text.

To compare the effectiveness of the guanylhydrazones as substrates for ADP-ribosylation by cholera toxin with that of other guanidino compounds reported in literature, we have carried out reactions with DEA-BAG under conditions similar to those reported with other compounds. The ADP-ribosyltransferase activity of cholera toxin with DEA-BAG is compared with the reported values of nicotinamide released in the presence of the guanidino compounds under identical conditions (Tables II and III).

Table II: Comparison of ADP-ribosyltransferase Activity of Cholera Toxin with Substituted (Benzylidineamino)guanidines and Alkylguanidines

compd	ADP-ribosyltransferase act. [nmol/(min·mg)][b]
	2.8 ± 0.4
	8.0 ± 0.9
	13.0 ± 1.0
	16.09 ± 0.78[a]

[a] The value is the nicotinamide released in the presence of 2 mM compound and is taken from Tait and Nassau (1984) under conditions exactly identical with those reported in this table. The ADP-ribosyltransferase activity will be less than the value reported as correction for NAD hydrolase activity is to be made. ADP-ribosyltransferase activity of cholera toxin with the substituted BAG derivatives was measured spectrophotometrically as described under Materials and Methods. The assay system contained 2 mM compound, 0.2 mM NAD, 200 mM phosphate, pH 6.5, 20 mM DTE, and 100 μg/mL cholera toxin. After 15 min, an incubation aliquot of 100 μL was transferred to the resin. [b] The values are averages of four estimations.

Table III: Comparison of ADP-ribosyltransferase Activity of Cholera Toxin L-Arginine Methyl Ester[b] and DEA-BAG[a]

phosphate concn (mM)	ADP-ribosyltransferase act. [nmol/(min·mg)]	
	2.0 mM DEA-BAG	75 mM LAME[b]
100	36.0	59.1
200	51.0	84.9

[a] Reactions were carried out under conditions similar to those described by Moss and Vaughan (1977) for ADP-ribosylation of LAME. The assay system contained 20 mM DTE, 2.0 mM NAD, 200 mM phosphate buffer, pH 7.0, 2.0 mM DEA-BAG, and 20.0 μg of cholera toxin in a total volume of 0.15 mL. After a 60-min incubation at 37 °C, the ADP-ribosyltransferase activity was measured spectrophotometrically as described under Materials and Methods. [b] The values are taken from Moss and Vaughan (1977) and correspond to the nicotinamide release in the presence of 75 mM LAME minus the nicotinamide released in the absence of LAME under identical conditions.

Use of Substituted (Benzylidineamino)guanidines in Structure–Activity Relationship Studies with Cholera Toxin. Substituted (benzylidineamino)guanidines with different substituents in the benzene nucleus have widely different substitutent constants and hydrophobicities. These compounds provide an ideal system for quantitative structure–activity relationship studies. The compounds were tested as substrates for the cholera toxin catalyzed ADP-ribosylation reaction. Initial velocities with all the BAG derivatives are measured spectrophotometrically as described under Materials and Methods. Kinetic parameters for all compounds are evaluated and tabulated in Table IV.

Consideration of Table IV indicates that electron-donating substituents are associated with increased k_{cat} values. The electron donating/withdrawing characteristics of the substituents are represented by the σ value for a substituent; $\sigma < 0$ indicates electron-donating capability while $\sigma > 0$ indicates

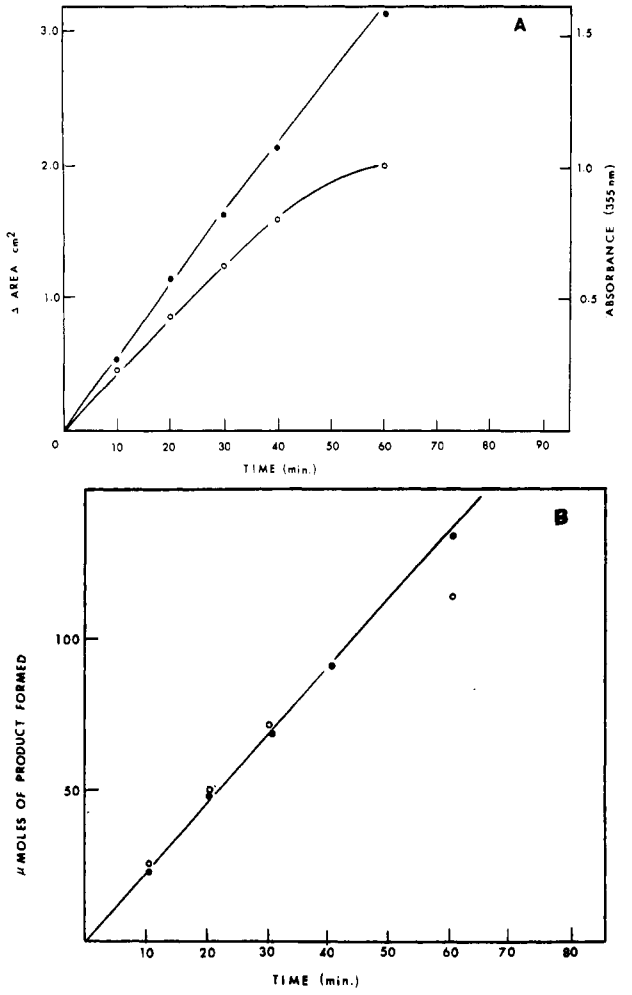


FIGURE 3: Time course of ADP-ribosylation of DEA-BAG as measured by spectrophotometric and HPLC methods. The assay system contained 10.0 mM NAD, 20.0 mM DTE, 50 mM Tris–acetate (pH 7.5), and 20 μg/mL cholera toxin A subunit at 30 °C. At the indicated time points aliquots of 100 μL are transferred to 150–200 mg of resin suspended in 2.0 mL of 20.0 mM phosphate (pH 7.4) and shaken well, and the resin is allowed to settle down. (A) The absorption of the supernatant is recorded at 355 nm (●). Control samples are set without cholera toxin. For HPLC analysis aliquots are transferred at the indicated time points to an equal volume of 10% TCA and filtered. HPLC was performed under conditions described under Materials and Methods. The change in area (○) was calculated from HPLC traces. (B) The absorption readings and areas in panel A are converted to amount of products formed.

electron-withdrawing ability. Regression analysis of the data indicates significant correlation of log k_{cat} and log (k_{cat}/K_m) with σ (Figure 4). The data are observed to be fit by

$$\log k_{cat} = [-0.37\ (\pm 0.07)]\sigma + 3.44\ (\pm 0.06)$$
$$r = 0.84,\ s = 0.281,\ F = 26.0,\ n = 13$$
$$\log (k_{cat}/K_m) = [-0.37\ (\pm 0.08)]\sigma + 6.05\ (\pm 0.06)$$
$$r = 0.81,\ s = 0.279,\ F = 20.3,\ n = 13$$

where r is the coefficient of correlation, s is the standard deviation in the regression, F is the goodness of fit statistic, and n is the number of compounds included in the correlation. Substitution of σ^+ for σ is detrimental to the correlation (not shown). Multiple regression analysis employing experimentally measured π values,[2] molar refractivity constants, steric constants, and values of π from the literature (Hansch & Leo, 1979) together with σ or σ^+ shows little improvement over the regression equations already given.

[2] π = log P_X − log P_H, where log P_X is the log of the octanol–water partition coefficient for the substituted (benzylidineamino)guanidines and log P_H is the log of the octanol–water partition coefficient for (benzylidineamino)guanidine.

Table IV: Kinetic Parameters for ADP-ribosylation of Substituted BAG Derivatives[a]

X[c]	σ[d]	$k_{cat} \pm$ SE[b] (10^3 s^{-1})	$K_m \pm$ SE (10^{-3} M)	$k_{cat}/K_m \pm$ SE (10^6 s^{-1} M^{-1})
2-(NO_2)	2.00	0.89 ± 0.10	3.14 ± 0.42	0.28 ± 0.02
4-Cl, 3-(NO_2)	0.94	1.04 ± 0.19	2.56 ± 0.56	0.40 ± 0.05
NO_2	0.79	0.71 ± 0.10	1.55 ± 0.28	0.45 ± 0.03
3-(NO_2)	0.71	0.77 ± 0.09	2.70 ± 0.43	0.28 ± 0.04
Br	0.23	3.48 ± 0.90	2.59 ± 0.74	1.34 ± 0.16
Cl	0.23	2.77 ± 0.28	5.35 ± 0.56	0.52 ± 0.02
H	0.00	2.87 ± 0.07	1.85 ± 0.07	1.55 ± 0.05
C_6H_5	−0.01	1.57 ± 0.52	0.48 ± 0.18	3.27 ± 0.52
$OC(CH_3)_3$	−0.20	4.00 ± 0.83	2.27 ± 0.50	1.76 ± 0.14
OCH_3	−0.24	4.33 ± 0.78	4.34 ± 0.90	0.99 ± 0.10
OC_2H_5	−0.27	5.85 ± 0.78	3.06 ± 0.51	1.92 ± 0.19
$N(CH_3)_2$	−0.83	5.68 ± 0.56	3.17 ± 0.38	1.79 ± 0.12
$N(C_2H_5)_2$	−0.90	5.64 ± 1.18	2.86 ± 0.72	1.97 ± 0.26

[a] Parameters calculated as described under Materials and Methods. [b] Standard error of the mean. [c] X denotes para substituents (position 4) of (benzylideneamino)guanidines, except where specifically described. [d] Values of σ taken from Hansch and Leo (1979). All values are for [e]para, except for 3-(NO_2), which is [e]meta, and 2-(NO_2), which is [e]ortho. The value for the 4-Cl, 3-(NO_2) derivative is the sum of the individual constants.

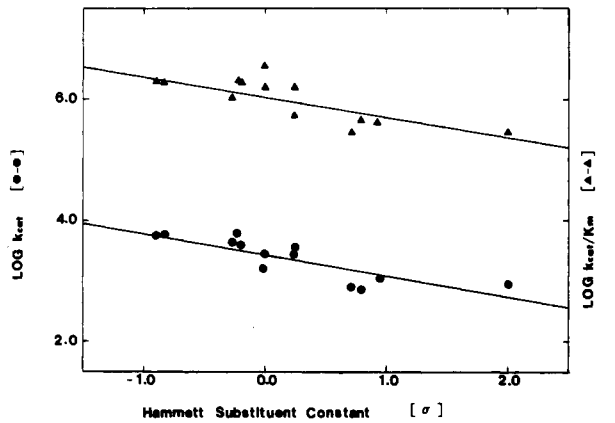


FIGURE 4: Correlation of the kinetic parameters with the physical characteristics of the compounds. Kinetic parameters were taken from Table IV. Values of σ were obtained from Hansch and Leo (1979).

The slope of the regression equation is denoted ρ. In the correlation of both log k_{cat} and log (k_{cat}/K_m), the value of ρ is <0. This is suggestive of the generation of an electron-deficient reaction center (March, 1985) in the transition state. This may explain the increased k_{cat} values in the presence of electron-donating substituents. If an electron-deficient (partly positive) condition is generated at the reaction site, electron donation from the ring may aid in the stabilization (formation) of the transition state.

The results of Table IV reveal that, despite the wide variations in the electronic and hydrophobic parameters of the different substituents, the K_m values for the compounds are not very different. Though electron-withdrawing groups, such as the nitro group at the para position, slightly lowered the K_m values and electron-releasing groups slightly enhanced the K_m values, there is no good correlation between the substituent constants and the K_m values.

Discussion

The results presented in this paper show the potential uses of guanylhydrazones in the quantitative assay and structure–activity relationship studies of guanidino group specific ADP-ribosyltransferases.

Quantitative assay methods for guanidino group specific ADP-ribosyltransferases have been reported earlier with use of ^{125}I-labeled guanyltyramine (Mekalanos et al., 1979; Watkins et al., 1985) and [(*p*-nitrobenzylidine)amino]-guanidine as model substrates (Soman et al., 1983, 1984b). The former method involves the synthesis and handling of a highly radioactive compound of short half-life. The latter method involves the use of high-performance liquid chromatography and is time-consuming. Though a spectrophotometric assay has also been suggested, the method is of limited applicability and has a number of limitations (Soman et al., 1983). The spectrophotometric assay method suggested here does not have any of the limitations and drawbacks mentioned for the other methods. The method works well with cholera toxin. We have also tested the applicability of the method for the measurement of ADP-ribosyltransferase activity in rabbit skeletal muscle homogenate and the sarcoplasmic reticulum, glycogen pellet, and sarcolemma preparations from pig skeletal muscle. The method works quite satisfactorily with the muscle homogenate and membrane preparation and gives results comparable to the HPLC analysis (Soman and Graves, unpublished results).

A larger number of guanidino compounds are reported as acceptors of the ADP-ribosyl group from NAD in reactions catalyzed by cholera toxin and other guanidino group specific ADP-ribosyltransferases. These include guanidine, arginine, agamatine, arginine methyl ester (Moss & Vaughan, 1977, 1978; Moss et al., 1983), substituted alkylguanidines (Tait & Nassau, 1984), and the guanylhydrazones (Soman et al., 1983, 1984; Graves et al., 1983). A direct comparison of the acceptor abilities of these different compounds has not been done so far. Because there is not a common method available for measuring the ADP-ribosyltransferase activity with all these compounds, a perfect comparison is rather difficult. With the majority of the guanidine or arginine derivatives, the activities are measured in terms of the abilities of these compounds to release nicotinamide. Because NAD hydrolase activities are also associated with cholera toxin and other enzymes (Moss & Vaughan, 1979; Moss & Richardson, 1978), the nicotinamide release is partly due to contributions from the NAD hydrolase activity. We have made an attempt to compare the efficiencies of the guanylhydrazones as ADP-ribosyl group acceptors compared to other guanidino compounds listed in literature. The results of Tables II and III suggest that DEA-BAG is at least 1.5–2 orders of magnitude more effective as substrate than arginine methyl ester and is as effective as the long-chain alkylguanidines reported by Tait and Nassau (1984).

Earlier studies using simple guanidino compounds suggested that the ADP-ribosyltransferase activity of cholera toxin is influenced by electronic and hydrophobic factors (Moss & Vaughan, 1977; Tait & Nassau, 1984). No detailed studies have so far been done to delineate the contributions of these factors on substrate binding and reaction rate. The results of this paper show that the binding of the BAG derivatives with the cholera toxin A subunit is not influenced to any significant extent by electronic and hydrophobic factors. All the compounds tested were aromatic and relatively hydrophobic. The observation that the K_m values for the guanidino compounds vary from 75 mM for arginine to 0.7–6 mM for the guanylhydrazones to $\sim$50 μM for iodinated guanyltyramine suggests that the binding of these molecules is significantly influenced by the physical characteristics of the guanidino compounds. Studies using a more extended series of compounds are required to find out what properties of the guanidino compounds are important in binding or the relative importance of various factors on the binding of these molecules. The structure–activity relationship studies performed here indicate that the cholera toxin catalyzed ADP-ribosylation of guanylhydrazones is influenced by electronic factors. Electron-releasing groups in the benzene nucleus enhance the pK_a of the guanidino groups of the guanylhydrazones and result in increased reactivity toward ADP-ribosylation by cholera toxin.

The correlation of log k_{cat} and log (k_{cat}/K_m) with σ allows insight into the chemical mechanism. The observation that $\rho < 0$ indicates the generation of an electron-deficient reaction center. The magnitude of ρ is insufficient to propose a fully charged site. This is indicative of a transition state in which there is partial bond formation and partial bond breakage of groups at the C-1 carbon of the ribose moiety. The generation of such a transition state is consistent with an S_N2 reaction mechanism in which the guanidino group of the substrate makes a nucleophilic attack on the C-1 carbon of the ribose moiety bonded to nicotinamide. Such an attack would cause an inversion at the C-1 carbon. Oppenheimer (1978) has shown that the cholera toxin catalyzed ADP-ribosylation of arginine produces the α-N-glycosidic anomer as the initial reaction product, which subsequently undergoes anomerization in solution. The guanylhydrazones bound to the enzyme may be deprotonated by proton abstraction by some basic functional groups on the protein. The deprotonated enzyme-bound guanylhydrazone then may make a nucleophilic attack on the C-1 carbon. Electron-donating substituents in the benzene nucleus enhance the pK_a of the guanidino group and also enhance the nucleophilicity, thereby facilitating the nucleophilic attack. This would explain the observed increase in reactivity with increase in electron-donating power of the substituents.

The molecular mechanism by which cholera toxin and *E. coli* enterotoxin cause the pathogenic state in host cells has been worked out recently (Johnson et al., 1978; Cassel & Pfeuffer, 1978; Gill & Merren, 1978). However, very little is known about the enzymology of the reactions catalyzed by these toxins. The ability of these toxins to act on small-molecular-weight guanidino compounds could be used to advantage in understanding more about the enzymology of the process. By extending the structure–activity relationship studies, by including compounds with different substituents in different positions of the benzene nucleus and other parts of the molecule, and also by including aliphatic analogues and other classes of guanidino compounds, it may be possible to get more information regarding the interactions of these guanidino compounds with the functional residues at the active site. Such studies will also give information regarding the mechanism of ADP-ribosylation processes. Eventually these studies might lead to the development of very effective affinity labels for the toxin active site and/or potential substrates or inhibitors for these toxins, which could be used as drugs against these toxins. Such studies are in progress in our laboratory.

Registry No. **1**, 3357-37-7; **2**, 30068-29-2; **3**, 13308-88-8; **4**, 37873-43-1; **5**, 13308-82-2; **6**, 82530-96-9; **7**, 102632-28-0; **8**, 100871-36-1; **9**, 61072-53-5; **10**, 38407-85-1; **11**, 102632-29-1; **12**, 90792-54-4; **13**, 102632-30-4; **14**, 102632-31-5; NAD–protein ADP-ribosyltransferase, 58319-92-9.

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Purification and Characterization of Three Distinct Glutathione Transferases from Mouse Liver†

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ABSTRACT: Three distinct glutathione transferases in the liver cytosol fraction of male NMRI mice have been purified by affinity chromatography and fast protein liquid chromatofocusing. These enzymes account for approximately 95% of the activity detectable with 1-chloro-2,4-dinitrobenzene as electrophilic substrate. Differences between the three forms are manifested in isoelectric points, apparent subunit molecular mass values, amino acid compositions, N-terminal structures, substrate specificities, and sensitivities to inhibitors, as well as in reactions with specific antibodies raised against glutathione transferases from rat and human tissues. The results indicate strongly that the three mouse enzymes are products of different genes. A comparison of the mouse glutathione transferases with rat and human enzymes revealed similarities between the transferases from different species. Mouse glutathione transferases have been named on the basis of their respective subunit compositions.

The glutathione transferases catalyze the conjugation of glutathione with numerous compounds carrying an electrophilic center (Chasseaud, 1979; Jakoby & Habig, 1980). They are also capable of binding nonsubstrate ligands, such as bilirubin and azo dye carcinogens (Smith & Litwack, 1980).

In many tissues, multiple forms of glutathione transferase are present (Mannervik, 1985). The different enzymes of rat liver (Jakoby & Habig, 1980; Mannervik & Jensson, 1982) and human liver (Kamisaka et al., 1975; Awasthi et al., 1980; Warholm et al., 1980, 1983) have been the subject of various studies, whereas the glutathione transferases in mouse liver have received less attention. In the mouse, strong interstrain

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