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The reaction between thiols and 8-azidoadenosine derivatives

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ABSTRACT

Thiols react at room temperature in dilute solution with 8-azidoadenosine and its nucleotides to give the corresponding 8-aminoadenosine derivatives. The reaction which takes place in the dark is base-catalysed and is particularly rapid when dithiols, e.g. dithiothreitol are used.

INTRODUCTION

8-Azidoadenosine nucleotides have been used recently as photoaffinity labels for a number of proteins[1-5]. During our investigations on the nucleotide binding sites of polynucleotide phosphorylase[6] and RNA polymerase[7], reducing conditions were required to maintain full activity of the enzymes. We found that a rapid reaction took place at room temperature in the dark between dilute aqueous solutions of 8-azido ADP(z^8ADP) or 8-azido ATP(z^8ATP) and dithiols such as DTT or DTE. Nitrogen was evolved and the ultraviolet spectra of the z^8Ado nucleotides rapidly changed to that of 8-aminoadenosine nucleotides. In order to maintain the enzymes in a reduced state, other thiols were tried and we observed that the reaction between z^8Ado nucleotides and monothiols, e.g. 2-mercapto-ethanol was very slow in aqueous solution at room temperature. We now report detailed investigations on the reaction between thiols and z^8Ado nucleotides.

MATERIAL AND METHODS

Alkaline phosphatase (E. C. 3. 1. 3. 1) and AMP were obtained from the Boehringer Corporation (London) Ltd. Tetramethylguanidinium azide was supplied by Lancaster Synthesis Ltd., Lancaster, U.K. Thiols were obtained from commercial sources and phenyl azide was the gift of Dr G. H. Dodd, University of Warwick.

8-AzidoAMP[1] - This was prepared from 8-bromoAMP[8] which had been purified by chromatography on DEAE-Sephadex A25 (HCO_3^- form, elution with a 0-0.15 M gradient of triethylammonium bicarbonate pH 8.7). The main alteration to the published procedure[1] was that tetramethylguanidinium azide was used to introduce the azido-group into the 8-position of the adenine ring. All manipulations with z^8AMP and its derivatives were carried out, where possible, in the dark. The z^8AMP was isolated after chromatography on DEAE-Sephadex A25 (HCO_3^- form, elution with a 0-0.15 M gradient of triethylammonium bicarbonate pH 8.7) as the mono(triethylammonium) salt in 78% yield. UV (pH 7) λ_{max} 282 nm, ε 12,900: lit[1] (pH 7.4) λ_{max} 281 nm, ε 13,300.

8-AzidoADP - This was prepared in 40% yield by the phosphoromorpholidate method[9] and was isolated as the tris(triethylammonium) salt after chromatography on DEAE-Sephadex A25 (HCO_3^- form, elution with a 0-0.25 M gradient of triethylammonium bicarbonate pH 8.7).

8-AzidoATP - This was synthesised from the monophosphate by the method of Michelson[10], and was isolated in 60% yield as the tris(triethylammonium) salt after chromatography on DEAE-Sephadex A25 (HCO_3^- form, elution with 0.1-0.5 M gradient of triethylammonium bicarbonate pH 7.5).

8-Azidoadenosine - Mono(triethylammonium) z^8AMP (15 mg) was incubated for 18 h at 37° in tris-HCl buffer (1 ml, 50 mM, pH 9.0) containing alkaline phosphatase (3.5 U). The incubation mixture was applied to a Sephadex G-50 column (30 x 1 cm) which was washed with water. The fractions containing UV absorbing material were pooled, evaporated to dryness and the residue crystallised from hot water to yield 7.4 mg (80%) z^8Ado m.p. 196° dec. (lit.[11] 226-229 dec.), UV (pH 1) λ_{max} 281.5 nm, ε 17,600 (pH 11), λ_{max} 281.5 nm, ε 13,500 (lit.[11] (pH 1), λ_{max} 281 nm, ε 17,300; (pH 11) λ_{max} 281, ε 13,500). Found, C, 39.61; H, 4.15; N, 36.4%: $C_{10}H_{12}N_8O_4$ requires C, 38.96; H, 3.92; N, 36.35%.

Reaction between thiols and azides - The reaction between z^8AMP and thiols was followed by the change in UV absorbance at 300 nm with time at 25° in a variety of buffers[12]. The reference cell in any determination contained all components of the reaction mixture except z^8AMP. Conditions used in the various experiments are given in the legends to the tables and figures.

Isolation of products from the reaction between z^8AMP and DTT - Dithiothreitol

TABLE 1

RELATIVE REACTION RATES OF THIOLS WITH 8-AZIDOAMP[a]

Thiol	Relative rate of reaction[b]
Dithiothreitol	100
Dithioerythritol	100
1, 2-Ethanedithiol	2.5
1, 3-Propanedithiol	269
2, 3-Dimercaptopropanol	6
2-Mercaptoethanol	0[c]
Thiophenol	0[c]
L-Cysteine	2

a) Reaction conditions: To 3 ml of 1 mM dithiol (2 mM monothiol) in MeOH at 25° was added triethylamine (50 μl), followed by bistriethylammonium z^8AMP (25 μl, 12.82 mM in MeOH).
b) Approx second order rate constant for DTT = 1.15 $M^{-1}s^{-1}$ at 25°.
c) Reaction not detectable under the conditions of this experiment.

TABLE 2

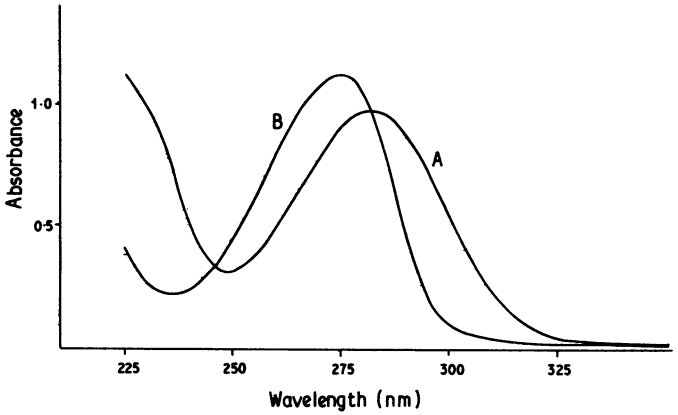
RELATIVE RATES OF REACTION OF DITHIOTHREITOL WITH AZIDES

Substrate	Relative rate[a]
z^8Ado	464
z^8AMP	100[b]
z^8ADP	83
phenyl azide	0[c]

a) Reaction conditions: To 0.1 M borate-phosphate buffer (3 ml, pH 8.9) at 25° was added 10 mM DTT (80 μl) and 0.5-0.7 OD_{300} unit of nucleoside or nucleotide. The second order rate of reaction of z^8AMP with DTT under these conditions is 7.41 : $M^{-1}s^{-1} \equiv 100$.
b) When borate-phosphate buffer/MeOH (1 : 1 v/v) was used as solvent the relative rate of reaction of z^8AMP was 30. Addition of diphenyl picryl hydrazyl up to 40 mole % had no effect on the reaction rate.
c) Phenyl azide (0.15 mM) was incubated in borate-phosphate buffer/MeOH (1 : 1 v/v) containing DTT (1 mM).

FIGURE 1

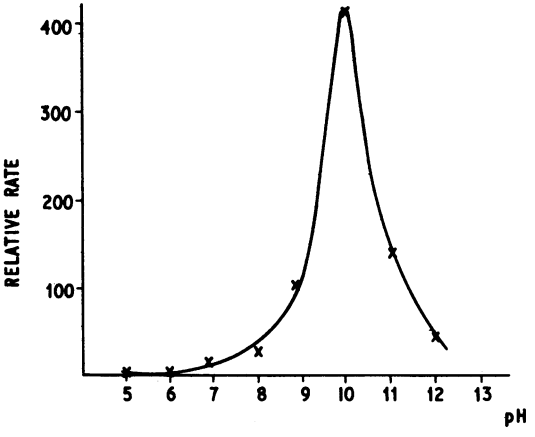
ULTRAVIOLET SPECTRUM OF 8-AZIDOAMP BEFORE AND AFTER ADDITION OF DTT



Spectra were determined in 0.1 M glycine-NaCl-NaOH buffer pH 9.1 before (A) and after (B) addition of DTT (80 µl, 10 mM) to 3 ml solution.

FIGURE 2

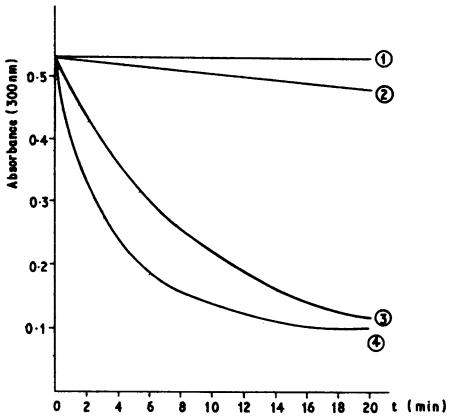
EFFECT OF pH ON THE RELATIVE RATE OF REACTION OF 8-AZIDOAMP WITH DTT



Reaction conditions: DTT (80 µl, 10 mM) and then monotriethylammonium z^8AMP (20 µl, 14.5 mM) were added to buffer (3 ml) and the reaction followed by the change in u.v. absorbance at 300 nm. Buffers used were:[12] pH 5-7 citrate-phosphate, pH 8-9 borate-phosphate pH 10-12 glycine-NaCl-NaOH. Apparent second order rate constant for the reaction at pH 8.9 = 7.41 $M^{-1}s^{-1}$ ≡ 100.

FIGURE 3

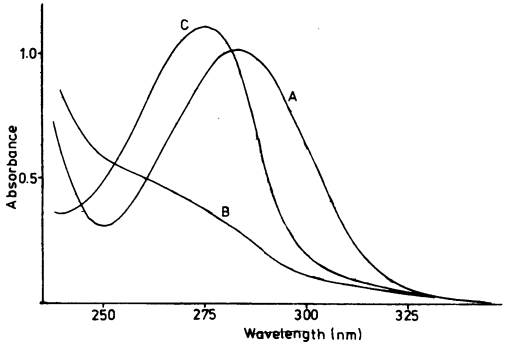
REACTION BETWEEN 2-MERCAPTOETHANOL AND 8-AZIDOAMP



Incubations, as described in Table 2, were performed in 0. 1 M glycine-NaCl-NaOH buffer pH 9. 1 with (1) 0. 52 mM (2) 10 mM (3) 100 mM 2-mercaptoethanol. Incubation with 0. 26 mM DTT (4) is shown for comparison.

FIGURE 4

PHOTOLYSIS OF 8-AZIDOATP IN THE PRESENCE AND ABSENCE OF MERCAPTOETHANOL



Spectra were determined in 0. 1 M Tris-HCl buffer (3 ml) pH 8. 0 as follows:

(A) Immediately after preparation of the 8-AzidoATP solution
(B) After a 5 minute photolysis
(C) After the addition of mercaptoethanol (75 μl, 100 mM) and photolysis for 5 minutes.

When mercaptoethanol (75 μl, 100 mM) was added to (A) and the solution left in the dark for 5 minutes there was no significant change in the spectrum. The photolyses were performed in quartz cuvettes at a distance of 10 cm from a Sylvania germicidal (GT85) lamp.

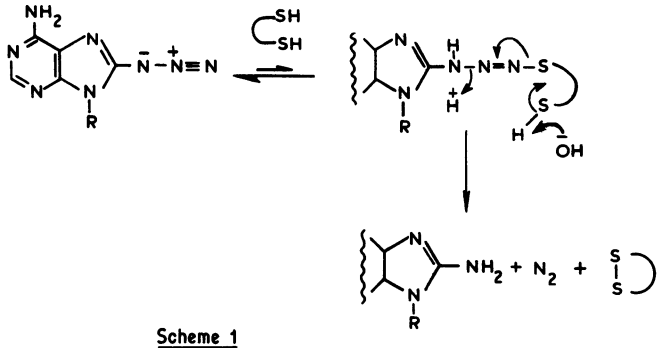
(33 mg, 0.2 mmole) was added in portions to a solution of bis(triethylammonium) z^8AMP (25 mg, 0.05 mmole) in 10 mM aqueous triethylammonium bicarbonate (5 ml, pH 8.7). Rapid evolution of gas occurred and the mixture was stirred in the dark for 18 h after which it was evaporated to dryness in vacuo. Excess triethylammonium bicarbonate was removed by repeated addition and evaporation of small amounts of methanol. A solution of the residue in water (10 ml) was applied to a column of DEAE-Sephadex A25 (HCO_3^- form, 20 x 0.75 cm) which was eluted with a 0-0.15 M gradient of triethylammonium bicarbonate pH 8.7. The fractions containing the major product were pooled, evaporated to dryness and excess triethylammonium bicarbonate removed as above to give 19 mg solid (80%), which ran as a single spot on tlc in a variety of solvents with the same R_F as authentic 8-aminoAMP prepared by hydrogenation of the azide.

The 8-aminoAMP was not purified further but was dephosphorylated as described above for z^8AMP, to give colourless crystals of 8-aminoadenosine m.p. 188-192° (lit.[11] 180-185°), UV (pH 1) λ_{max} 269, ε 13,200; (pH 11) λ_{max} 274, ε 16,400: (lit.[11] UV (pH 1) λ_{max} 270 nm, ε 13,500; (pH 11) λ_{max} 273, ε 16,400. Mass spectrum m/e M^+ 282, $(B+H)^+$ 150. Found, C, 42.48; H, 5.36; N, 29.7%. $C_{10}H_{14}N_6O_4$ requires C, 42.55; H, 5.00; N, 29.8%.

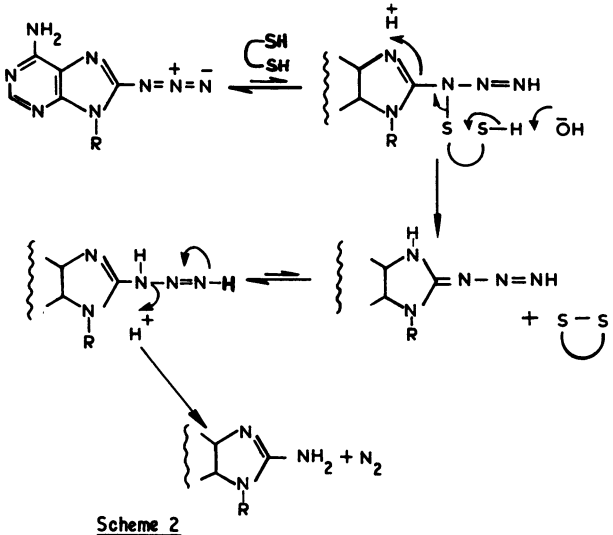
RESULTS AND DISCUSSION

The reduction of azides by thiols has been reported previously[13, 14]. However, forcing conditions (e.g. 100° for several hours) were required for the reaction to go to completion. We have observed that a rapid, base-catalysed reduction of 8-azidoadenosine and derived nucleotides by dithiols can occur in aqueous solution at room temperature. The corresponding reaction of z^8AMP with monothiols is very slow, although it can be observed at high concentrations of monothiol. The reaction between dithiols and aromatic azides, e.g. phenyl azide, is also very slow under our conditions. Free radicals do not appear to be involved if the reaction is carried out in the dark as addition of diphenyl picryl hydrazyl has no effect on the reaction rate. The photochemical reduction of azides by thiols may well involve free radicals[13]. We have observed that while there is no reaction in the dark between z^8ATP and 2.5 mM 2-mercaptoethanol, 8-aminoATP is formed when the mixture is irradiated with ultraviolet light.

Two alternative mechanisms can be suggested for the dark reaction. In the first there is a rapid, reversible attack by the dithiol on the terminal (γ) nitrogen



Scheme 1



Scheme 2

of the azide followed by intramolecular cyclisation and extrusion of nitrogen to produce the cyclic disulphide and 8-aminoadenosine. This is analogous to the reaction between phosphines and phenyl azides where the initial product is an adduct formed by attack of the phosphine on the terminal nitrogen atom of the azide[15]. In the second mechanism the dithiol attacks the α-nitrogen atom of the azide, again in a rapid reversible reaction. Intramolecular cyclisation of the dithiol followed by elimination of nitrogen would give 8-aminoadenosine.

We have observed the formation in the reaction between 1, 3-propane dithiol and z^{8}AMP of a non-nucleotidic, weakly u. v. absorbing compound (λ_{max} 329 nm) which is presumably the cyclic disulphide of 1, 3-propane dithiol as this has been reported to have a weak u. v. absorption at 330 nm[16]. The rates of intramolecular formation of cyclic disulphides should depend on the sizes of the rings formed.

Thus, ethane 1, 2-dithiol would be expected to form a disulphide more slowly than propane 1, 3-dithiol which would be one explanation of the difference in reaction rates of these two dithiols with z^8AMP. The formation of disulphides from either azide-monothiol adduct would have to occur by an intermolecular process and hence this should be considerably slower than the corresponding intramolecular reaction. Presumably the initial equilibria lie in favour of the reactants and hence the reaction between z^8AMP and monothiols can be accelerated by increasing the concentration of monothiol.

The rate of reaction between z^8AMP and DTT or 2-mercaptoethanol passes through a maximum at pH 10, indicating that the anion of the thiol must be the species which reacts initially with the azido group and that competing reactions must occur at higher pH. Ionisation of the α-NH group (Scheme 1) or the γ-NH (Scheme 2) could be reactions which compete with disulphide formation. The rapid reduction at pH values above neutrality of 8-azido adenosine nucleotides by DTT and other dithiols might interfere with the photoaffinity labelling by those nucleotides of enzymes which require reducing conditions to maintain activity. In these cases, a monothiol such as 2-mercaptoethanol should be used in low concentration to avoid rapid deactivation of the photoaffinity label.

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