THE REACTION OF AN ORGANOMERCURY COMPOUND WITH A NUCLEOSIDE PHOSPHOROTHIOATE

Implications for the prospect of electron microscopic nucleic acid sequencing

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1. Introduction

The proposal of determining nucleic acid sequences by base-specific labeling with heavy elements and subsequent electron microscopic observation has been formulated [1], but has not been put into practice. It is generally considered that two types of problems must be resolved before this can be done.

(i) A method must be available for labeling each of the four bases with high specificity.
(ii) Electron microscopists must be able to locate single atoms or clusters of atoms with sufficient confidence.

We are concerned here with the former of the two problems. The original approach consisted in a search for base-specific reactions of nucleic acids with electron-dense reagents [2], but it has proven difficult to modify one out of four bases exclusively and quantitatively. An alternative approach is to introduce the label by in vitro enzymatic replication or transcription of the nucleic acid using one heavy element-modified and three unmodified nucleoside triphosphates as substrates. Mercurated pyrimidine nucleotides were proposed for this purpose [3] but proved not to be stable enough [4]. Nucleoside 5′-*O*-(1-thiotriphosphates) was used [5] as modified building blocks for the in vitro synthesis. The sequence-specific label is obtained by reaction of a terpyridine platinum salt with the phosphorothioate groups of the resulting polymer. This approach looks promising because the same derivatives can be used of each of the four bases and because DNA replicas with one of the nucleotides faithfully substituted by the phosphorothioate analog were obtained [6].

Organomercury compounds of the type RHgX are known to have a high affinity for thiols and therefore seemed possible candidates as electron dense reagents for phosphorothioate groups. If such a reaction takes place, the obvious criterion in deciding whether it will be quantitative under given circumstances is the equilibrium constant. Although K_a values can be found in the literature for the reactions of RHgX compounds with several thio-analogs of nucleic acid bases [7], nucleosides and polynucleotides [8], and with s^4U-containing tRNAs [9,10], no such data are available for reactions of heavy metal compounds with nucleoside phosphorothioates. We show here that adenosine 5′-phosphorothioate (p_sA) reacts with 4-(*p*-sulphophenylazo)-2-mercuriphenol (SAMP) with a K_a of 1.6×10^7 l/mol.

2. Materials and methods

p_sA was in part synthesized according to [11] and in part purchased from Serva Feinbiochemica, Heidelberg. SAMP was synthesized [12] by Dr Joniau, IRC, University of Leuven, Kortrijk, who kindly made available a sample for our experiments.

The choice of SAMP as the mercurial for examining

Abbreviations R, R′, organic radicals, X, anion; s^4U, 4-thiouridine, p_sA, adenosine 5′-phosphorothioate, SAMP, 4-(*p*-sulphophenylazo)-2-mercuriphenol

the reaction with p_sA deserves some comment. Association constants for binding of mercurials to thioanalogs of nucleic acid constituents [7,8] are usually computed by spectrophotometric methods, the reaction causing a shift in the spectrum of the thiobase. Binding of a mercurial to a nucleoside phosphorothioate does not affect the nucleotide spectrum. However, if one choses a reagent where the mercury is bound to a chromophore displaying an absorption maximum outside the spectral range of the nucleic acid bases, one can, under proper conditions, observe a shift in the mercurial spectrum.

As illustrated in fig.1, SAMP is subject to an acid–base equilibrium [12], the acid form showing an absorption maximum at 358 nm and the basic form at 442 nm. The pK_a value is 7.53, but rises to higher values when a thiol is bound, to an extent depending on the thiol used. In the presence of p_sA, we found a new pK_a of 8.9, determined spectrophotometrically [12]. If we now work at a pH between the pK_a values of unmodified SAMP and

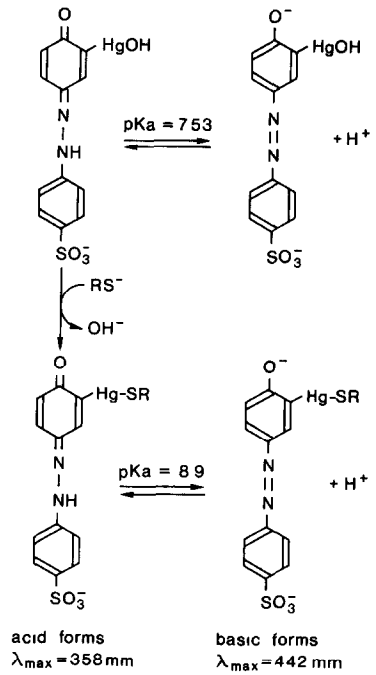

Fig 1 Acid–base equilibrium of SAMP and of the derivative resulting from reaction with p_sA The pK_a value for thioderivatives of SAMP varies with the nature of RSH [12] and was found to have the value 8.9 if $RS^- = p_sA$.

of its p_sA derivative, e.g., at pH 8, then binding of the p_sA will result in a shift from the basic to the acidic form, and will be observed as a spectral shift.

The reaction between SAMP and p_sA was studied by the method of continuous variation [13–15]. A series of mixtures was prepared containing 0.05 M sodium borate buffer (pH 8) and varying amounts of p_sA and SAMP with total conc. 1.5×10^{-5} M for the two compounds. Absorbances were measured at 350, 360, 410, 420, 430, 440 and 450 nm in a Zeiss PMQ3 spectrophotometer at room temperature. Quartz cuvettes of 5 cm light path were used. The K_a was computed as detailed in [15].

3. Results and discussion

The continuous variation plots of the absorbances of SAMP–p_sA mixtures shown in fig.2 demonstrate that the compounds react in a 1:1 ratio. From 7 plots, constructed for the wavelengths mentioned above, the following mean association constant was calculated:

$$K' = 1.6 \times 10^7 \text{ l/mol} \pm 0.4 \times 10^7 \text{ l/mol}$$

This value is slightly larger than the highest binding constant reported [7] for the reaction of a thiobase with a mercurial. It may be appropriate to recall that our value, as well as other binding constants reported [7–10] for reactions between nucleic acid thio-constituents and mercurials is not the equilibrium constant K of the reaction

$$RS^- + R'Hg^+ \rightleftharpoons RSHgR' \qquad (1)$$

with

$$K = \frac{(RSHgR')}{(RS^-)\,(R'Hg^+)}$$

but a conditional equilibrium constant [16].

$$K' = \frac{(RSHgR')}{[C_{RS^-} - (RSHgR')]\;[C_{R'Hg^+} - (RSHgR')]} \qquad (2)$$

where $(RSHgR')$ is the equilibrium concentration of the compound formed.

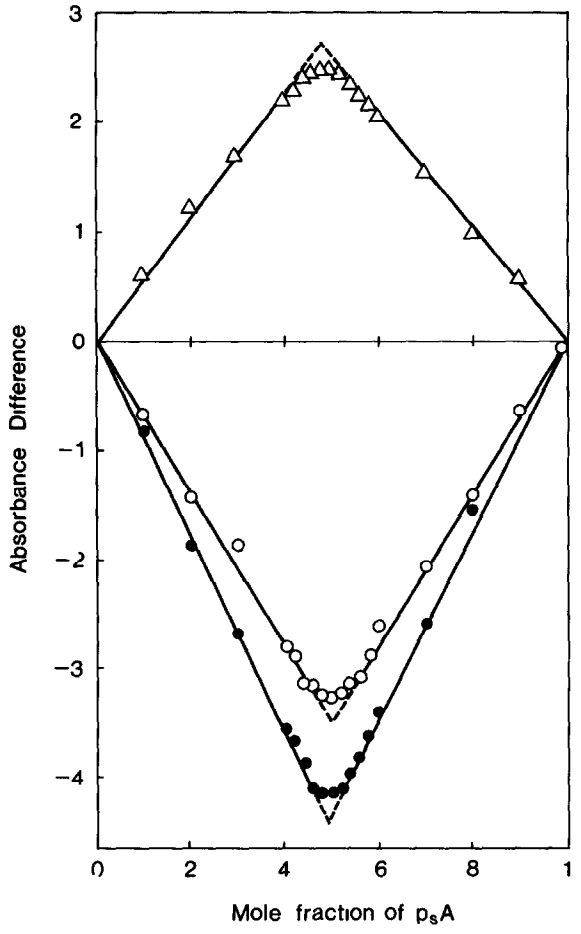

Fig 2. Continuous variation plots of SAMP–p_SA mixtures. The total concentration of SAMP + p_SA was 1.5×10^{-5} M for all mixtures. Plotted in ordinate is the difference between the absorbance measured after mixing and the sum of the absorbance due to SAMP and p_SA before mixing. The binding constant is computed [15] from the experimental absorbance difference (solid lines) and the extrapolated difference (broken lines) The plots shown are those constructed for A_{350} (△——△), A_{410} (○——○) and A_{440} (●——●) measurements

C_{RS^-} and $C_{R'Hg^+}$ are the analytical concentrations of the thio-derivative and the mercurial. Since hydrogen ions compete with $R'Hg^+$ for reacting with RS^-, while OH^-, Cl^- and other ions can compete with RS^- for reacting with $R'Hg^+$ [17], it follows that the equilibrium of reaction (1) will be shifted to the left to a degree depending on the pH of the solution and the concentration of salts present. The K' value allows us to compute how quantitative the conversion of RS^- to RSHgR' is, without considering equilibria of side reactions, but only in the particular ionic environment used

The K' as determined above prevails at pH 8, i.e., near neutrality, and in the absence of ions such as Cl^- that show a strong affinity for mercurials. Such conditions should be ideal for the labeling of base-specifically phosphorothioate-substituted nucleic acids with mercurials. Assuming the equilibrium constant for the binding of SAMP to thiophosphodiesters in a polynucleotide to be of the same magnitude as that for binding to thiophosphomonoesters, we can estimate the completeness of the conversion of a modified nucleic acid to the mercury derivative. This has been done in table 2 for nucleic acid concentrations of 10^{-5}–10^{-3} M, likely to be used in preparations for electron microscopy [2], to which 1–10 equiv. SAMP/equiv. phosphorothioate is added. It shows that even at low nucleic acid concentration, and if the excess mercurial over phosphorothioate were kept low in order to minimize the presence of

Table 1
Completeness of nucleoside phosphorothioate conversion into its mercury derivative

Mercurial equiv. added	Fraction of phosphorothioate residues binding mercury at a phosphorothioate concentration of		
	10^{-5} M	10^{-4} M	10^{-3} M
1	0.924	0 975	0.992
1.1	0.957	0.994	0 9994
1.5	0.987	0.9987	0 99987
2	0 994	0 9994	0.99994
5	0.998	0 9998	0.99998
10	0 999	0.9999	0 99999

The fraction of phosphorothioate residues binding mercury is computed for solutions 10^{-5}, 10^{-4} and 10^{-3} M in phosphorothioate to which 1–10 equiv. mercurial have been added, by substituting the following values in eq (2)

$C_{RS^-} = 10^{-3}$ M, 10^{-4} M or 10^{-5} M

$C_{R'Hg^+} = 1 \times C_{RS^-}, 1.1 \times C_{RS^-}, \ldots, 10 \times C_{RS^-}$

$K' = 1.6 \times 10^7$ l/mol

and solving for $(RSHgR')/C_{RS^-}$

background mercury atoms in the preparation, conversion of phosphorothioate moieties to mercury derivatives would be fairly complete.

It seems worthwhile to mention a potential advantage of mercurials over other phosphorothioate reagents such as platinum compounds [5]. The binding constants of different mercurials for an anion are of the same magnitude regardless the exact nature of the organic moiety R in RHg^+ [18], so that it may be assumed that the high affinity of SAMP for phosphorothioate is a general property of monoorganomercurials. On the other hand many mercurials are volatile at ambient temperature and soluble in organic solvents. This raises interesting possibilities for preparative techniques. The nucleic acids with base-specific phosphorothioate substitution could be spread on a thin film support on a microscope grid according to one of the existing methods [2]. After drying, the grid could be exposed to vapors of a volatile reagent such as CH_3HgOH. Excess mercury bound non-specifically to the support could be chased by slightly heating the sample under vacuum. Alternatively, the staining could be performed by dipping the grid into a solution of mercurial in an organic solvent, the hydrophilic nucleic acid remaining undisturbed, and excess mercurial being washed away in pure solvent. Finally, it might be possible to enhance electron microscopic contrast by using as labels RHgX compounds containing additional heavy atoms, such as iodine, in the organic moiety. A polymetallic heavy atom label for s^4U in tRNA has been proposed [19] but this may pose problems for an RNA containing many phosphorothioate residues because it has several binding sites.

In conclusion, we may state that the combination of preparing base-specifically phosphorothioate-substituted copies of nucleic acids, and of labeling them with compounds of the type RHgX, now seems the most promising approach to the preparative aspect of electron microscopic nucleic acid sequencing.

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