Dynamic aspects of affinity labelling as revealed by alkylation and phosphorylation of pancreatic ribonuclease with reactive deoxyribodinucleotide derivatives

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Affinity labelling of pancreatic RNase with 4-(*N*-2-chloroethyl-*N*-methylamino)benzylamide and (N→P) *N*-methylimidazolide of d(pTpA) results in the formation of monomodified enzyme derivatives retaining partially enzymatic activity. These data together with some cases described in the literature are considered as suggesting the dynamic nature of the enzyme-reagent complex represented by a set of states differing in the probability of intra-complex reaction. In particular, modification may proceed in a low probability state with an especially favorable mutual orientation of reagent and some protein residue remote from the active site of the enzyme resulting in the removal of the covalently attached reagent moiety from the active center.

Affinity labeling *Biopolymer dynamics* *Reactive dinucleotide derivative* *Pancreatic RNase*

1. INTRODUCTION

Affinity labelling of biopolymers is usually considered as being a reaction within a rather rigid complex of biopolymer with reagent recognized by the active site of the biopolymer. The commonly accepted criteria for affinity labelling and the equations used to treat the kinetics of the process are derived on the basis of this assumption [1–3]. Accordingly one definite group of biopolymers or a few adjacent groups have to be modified. The 'addressing' part of the reagent is covalently fixed in the vicinity of the active site thus leading to inactivation of biopolymer.

However, due to the conformational mobility of both biopolymer and recognized ligand it seems more likely to consider the complex as a dynamic system represented by a number of states with different conformations of the biopolymer and combinations of the contact points between partners Reaction should proceed preferentially within the states with the most favorable mutual orientation of the reagent and one of the potentially reactive groups of the biopolymer. These states are not necessarily identical with either the main functional states of a similar complex with the natural ligand (e.g. the state of the enzyme-substrate complex advantageous for catalysis) or the most populated states. Therefore, modification may proceed at a number of non-adjacent points of the biopolymer including points rather far away from the active site. The low probability of approach of these groups to the reactive moiety of the reagent within the complex may be compensated by favorable mutual orientation of the reacting fragments. In this case, the adduct formed may permit a much more probable conformation thus removing the attached reagent from the active site and, therefore, activity may be partially retained. Experimental data of this kind have already ap-

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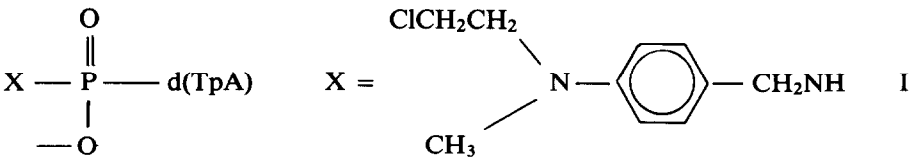
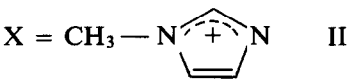
peared in the literature, some of which will be mentioned in section 4. However, the problem was never attacked systematically. To start this kind of investigation it is necessary to elaborate some sufficiently available and simple system permitting accumulation of a large set of quantitative and structural data. It is essential to deal with a biopolymer of known tertiary structure for correct interpretation of results.

Here, we demonstrate that affinity labelling of





pancreatic RNase (EC 3.1.27.5) may be a good candidate for such a system. In particular, we have found that alkylation of RNase A with reagent I and phosphorylation with reagent II, derivatives of deoxyribooligodinucleotide d(pTpA), result in the formation of adducts retaining enzymatic activity. In the case of alkylation with reagent I at least 4 different monomodified forms of the enzyme were found.

2. MATERIALS AND METHODS

Commercial pancreatic RNase was purified according to [4] to 97% homogeneity as revealed by $A_{280}^{0.1\%} = 0.71$ [5]. 4-(*N*-2-Chloroethyl-*N*-methylamino)benzylamide of d(pTpA) (II) was prepared as described in [6]. The conversion to 4-(*N*-2-hydroxyethyl-*N*-methylamino)benzylamide (Ia) was performed according to [7].

To prepare (N→P) 1-methylimidazolide of d(pTpA) (II), a mixture of the Et_3N salt of d(pTpA) (5 μmol) and *N*-methylimidazole (30 μmol) was treated in 0.1 ml anhydrous dimethylformamide with a mixture of 2,2′-dipyridyldisulfide and triphenylphosphine (15 μmol of each) for 10 min at room temperature. The product was precipitated with 0.7 ml of 2% $LiClO_4$ in acetone and the precipitate collected and washed with acetone (2 × 0.7 ml) and ether (0.7 ml). The content of reactive derivative (II) in the specimen was estimated to exceed 90% by conversion to d(pTpA) amide, by treatment with a 10-fold excess of aqueous tetramethylenediamine. The ratio amide: d(pTpA) was measured by microcolumn ion-exchange chromatography. Amide was identified by quantitative conversion to d(pTpA) by mild acid treatment (0.05 M HCl, 2 h, 40°C).

The enzymatic activity of native and modified RNase was followed by measuring the initial rate of hydrolysis of cCMP [8] or that of poly(U) [9]. The A_{280}/A_{260} ratios used for UV identification of modified forms of enzyme were calculated using the sums of molar extinction coefficients ($l \cdot mol^{-1} \cdot cm^{-1}$) for: RNase, $E_{260} = 5000$, $E_{280} = 5400$; reagent I, $E_{260} = 38700$, $E_{280} = 12500$; d(pTpA), $E_{260} = 24000$, $E_{280} = 9030$.

3. RESULTS

Pancreatic RNase is known to be a DNA-binding protein [10,11]. Recently, it was shown that all 16 dideoxyribonucleotides are competitive inhibitors of the enzyme [12]. The alkylating derivative of d(pTpT) similar to I was demonstrated to perform affinity labelling of RNase [8].

The unreactive analog of I, Ia, was found to be a competitive inhibitor of RNase with $K_i = 2.9 \times 10^{-4}$ M, rather close to that of d(pTpA) (7×10^{-4} M). Thus, the attacked group does not deprive the dinucleotide of its affinity for RNase. Incubation of the enzyme with reagent I results in partial inactivation of RNase as seen in fig.1. The dinucleotide d(pTpA) protects against inactivation according to one of the main criteria of affinity labelling.

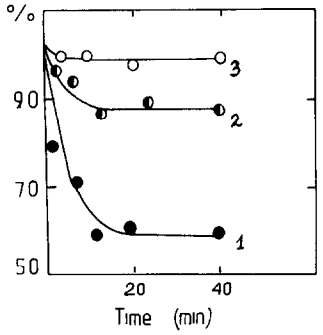


Fig.1. Kinetics of inactivation of 2×10^{-5} M RNase by 5×10^{-4} M I (0.1 M NaCl, 0.1 M Tris-HCl, pH 7.2, 25°C) in the absence (1) and presence of 2.1×10^{-3} M d(pTpA) (2) and 3.5×10^{-4} M Ia (3). Activity was determined following hydrolysis of cCMP.

Fig.2 shows the results of chromatography of the alkylated enzyme on CM-cellulose. Excess reagent was preliminarily separated by gel filtration on a Sephadex G-25 column. Besides unreacted RNase (A_{280}/A_{260} = 1.73), at least 4 peaks corresponding to monomodified enzyme (expected A_{280}/A_{260} = 0.5) are seen. There is no peak corresponding to doubly modified enzyme (A_{280}/A_{260} = 0.42), although, in total, the level of modification is 0.7 mol/mol. This is an additional argument in favor of the specificity of the reaction. Homogeneity of peak 1 was checked by tryptic hydrolysis. The respective profile is given in fig.3. Mainly one peptide exhibits a significant A_{260}

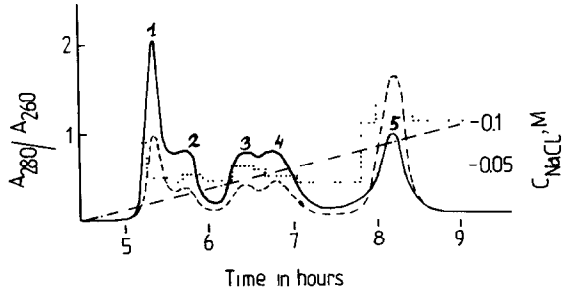


Fig.2. Chromatography of the products of the reaction between 3.7×10^{-3} M RNase and 6.4×10^{-3} M I (0.2 M NaCl, pH 7.0, 40°C, 7 h) on a CM-cellulose column (9.5 × 190 mm). Elution was carried out in 5 mM Tris-HCl, pH 8.0, with a linear NaCl gradient at a flow rate of 0.4 ml·min^{-1}. (——) A_{260}, (– – –) A_{280}, (···) A_{280}/A_{260}.

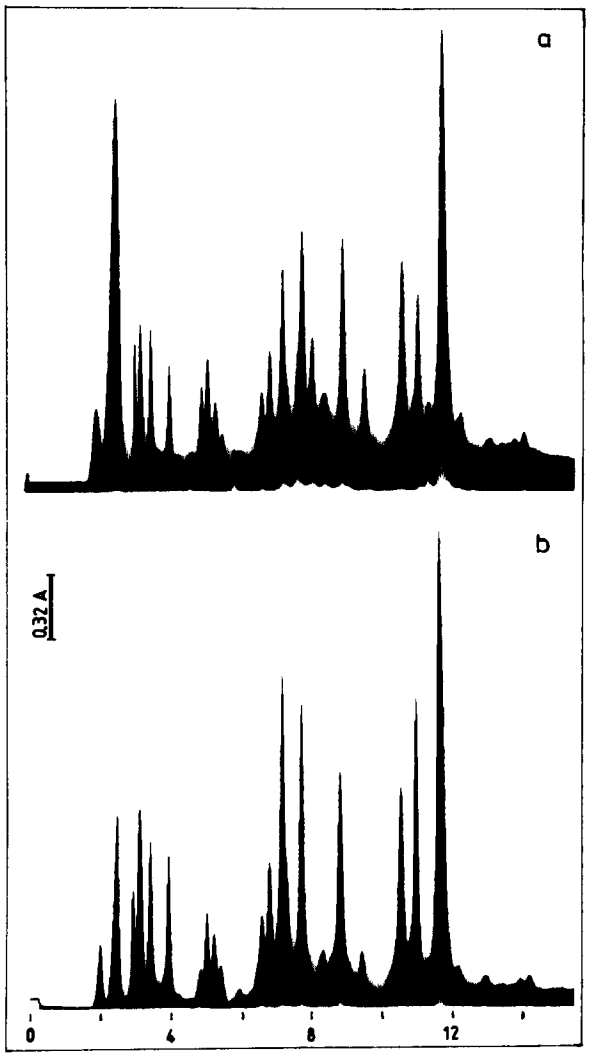


Fig.3. Microcolumn chromatography at λ 210 nm (dark area) and 260 nm (white area) of the peptides obtained by tryptic digestion of RNase derivative (peak 1, fig.2) (a) and non-modified RNase (b) on a Nucleosil 5-C_{18} column (2 ×62 mm). Elution was carried out in 0.1% CF_3COOH, pH 2.0, with an acetonitrile gradient (0–50%) at a flow rate of 100 μl·min^{-1}.

value expected for modified peptides. The amino acid composition of this peptide corresponds to the C-terminal peptide His_{105}–Val_{124}. All peaks corresponding to monomodified enzyme exhibit definite RNase activity as shown in table 1.

The lack of complete inactivation of RNase with derivative I could be due to flexibility of the spacer

Table 1

Enzymatic activity of RNase derivatives

Number of CM-cellulose chromatography peaks (fig.2)	Activity (%)	
	Substrate: cCMP	Poly(U)
1	23	94
2	46	88
3	64	100
4	60	100
5	100	100

group separating the dinucleotide moiety and the reactive C-Cl fragment. Therefore, we have studied the interaction of RNase with reagent II lacking any spacer. This type of reagent is described in this paper for the first time. They possess powerful phosphorylating ability with respect to aliphatic amines. At the same time, these reagents are rather stable in aqueous solution, the half-time of hydrolysis being about 150 min. A detailed description of the chemical properties of

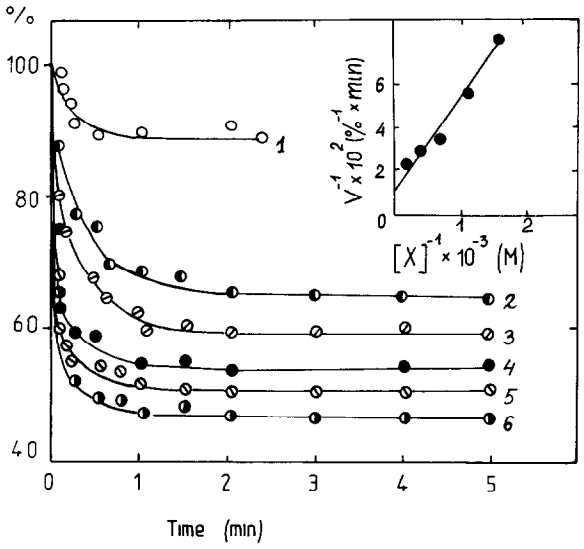


Fig.4. Kinetics of inactivation of RNase (4×10^{-5} M) by II: 4×10^{-4} M (2); 8×10^{-4} M (3); 1.2×10^{-3} M (4); 2×10^{-3} M (5) and 4×10^{-3} M (6), 0.2 M NaCl, 25°C. Curve 1 denotes 4×10^{-4} M reagent and 7×10^{-4} M d(pTpA). Inset: double-reciprocal plot of the dependence of the initial rate of inactivation on initial concentration of I (X).

(N→P) *N*-methylimidazolides of mono- and oligonucleotides will be given elsewhere.

The kinetics of inactivation of RNase with varying concentrations of II is presented in fig.4. In the inset of the same figure, the dependence of the initial rate of inactivation on the initial concentration of II is presented. The dissociation constant of the complex of enzyme with II is estimated as 10^{-3} M, quite close to that of reagent I and d(pTpA) itself. 0.7 mM d(pTpA) efficiently protects against inactivation. Thus, the essential quantitative criteria of affinity labelling are fulfilled.

Chromatographic separation of the reaction mixture on the CM-cellulose column results in the appearance of only one peak with A_{280}/A_{260} = 0.64, close to the calculated value for an equimolar RNase-d(pTpA) mixture. Thus, the absence of a spacer group abolishes multiplicity of the labelling. However, again, the product retains significant enzymatic activity (8% in the hydrolysis of cCMP, 50% in the hydrolysis of polyuridylate).

4. DISCUSSION

The data presented show that the modification of pancreatic RNase with reactive deoxyribodinucleotide derivatives exhibits some essential features expected from consideration of the dynamics of the process. Certainly, they do not prove the reliability of this consideration, and systematic quantitative investigation of the problem is needed. At the same time, our data indicate that the described system is a promising candidate for such an investigation. Some other systems described in the literature may be considered in a similar way. We shall restrict ourselves to a few examples.

The significant distortion of the ATP γ-*p*-azidoanilide fragment covalently attached to rabbit muscle creatine kinase by photoaffinity labelling was suggested to explain the retention of the affinity of modified enzyme towards ADP and ATP [13]. The ATP fragment covalently bound to *Escherichia coli* phenylalanyl-tRNA synthetase via γ-phosphate by affinity labelling with adenosine 5′-trimethaphosphate does not influence the affinity of phenylalanine and phenylalaninol, whereas free ATP significantly enhances this affinity. These data were explained as a result of displacement of the ATP fragment covalently

bound to enzyme from the active center [14].

Affinity labelling of multisubunit systems – ribosomes [15] and RNA polymerase [16] often results in the simultaneous modification of several different subunits. These results are often considered as suggesting the vicinity of the intersubunit area and the active site of the system. However, it may happen that the internal motion of subunits or their domains leads to occasional favorable approach of some subunits to the reactive group of the label followed by chemical reaction. Therefore, the dynamic aspects should not be neglected in the interpretation of the data obtained in affinity labelling experiments.

At the same time it is seen that both the points and the level of affinity labelling reflect in some way the dynamic behavior of the macromolecular system and may serve as a tool for the investigation of the dynamics of biopolymers.

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