The estimation of affinity constants for the binding of model peptides to DNA by equilibrium dialysis

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ABSTRACT
The binding of lysine model peptides of the type Lys-X-Lys, Lys-X-X-Lys and Lys-X-X-X-Lys (X = different aliphatic and aromatic amino acids) has been studied by equilibrium dialysis. It was shown that the strong electrostatic binding forces generated by protonated amino groups of lysine can be distinguished from the weak forces stemming from neutral and aromatic spacer amino acids. The overall binding strength of the lysine model peptides is modified by these weak binding forces and the apparent binding constants are influenced more by the hydrophobic character of the spacer amino acid side chains than by the chainlength of the spacers.

INTRODUCTION

There is no doubt that salt-like linkages between the negatively charged phosphate groups of the sugar phosphate backbone and the positively charged amino groups of the basic amino acids contribute most to the binding of histones to DNA.

However hydrogen bonding between nucleic acid bases and amino acid residues, hydrophobic type interaction of "apolar" amino acid side chains with the DNA surface and intercalation of aromatic amino acids into the DNA helix are also discussed as possible contributors to the overall binding of histones to DNA.

Because of the rather complicated structure of the nucleohistone complex, some workers have replaced the histones by amino acids or small peptides, when doing binding studies in order to get models which are easier to interpret. The goal of this type of investigation has been to provide evidence for the contribution of particu-
lar amino acids, within the peptide chain, to the overall binding to DNA.

With this in mind and taking for granted that the attachement of basic peptides to DNA is mainly by means of the protonated sidechain of the basic amino acids incorporated into the peptide, model peptides have been prepared which consist of a maximum of 5 units. These are characterized by the general formulas, Lys-X-Lys, Lys-X-X-Lys, and Lys-X-X-X-Lys, where X stands for the amino acids glycine, alanine, leucine and tyrosine. In addition di-lysine and diarginine have been synthesized.

Binding studies between DNA and the model peptides mentioned above are helpful in answering the following questions:

1. Is it possible to distinguish between the strong electrostatic forces of the protonated lysine amino groups and the expectedly weak forces generated by neutral and aromatic amino acids?

2. What influences do varying distances produced by neutral and aromatic amino acid spacers of different chain length between the terminal lysine side-chain functional groups, exert on the value of the binding constant?

In order to check if the base composition and distribution of the DNA used for the binding studies has some influence of the binding constants, we also conducted comparative studies with Lys-Ala-Lys and DNA from different sources (calf thymus = CT; Micrococcus lysodeictius = ML).

MATERIALS AND METHODS

Peptide synthesis

The peptides used for the binding studies were synthesized by the Merrifield technique\textsuperscript{17,18}. The amino acid attached last to the peptide was always \textsuperscript{14}C-labelled so that the radioactivity could directly be used to assess the concentration of the free peptide during the binding studies.

Some of the t-butoxycarbonyl-amino acids (Boc-amino acids) used were commercial products (Merck, Darmstadt), others were prepared in our laboratory. When preparing \textsuperscript{14}C-Boc-lysine, 250 \textmu Ci of \textsuperscript{14}C-L-lysine-HCl, uniformly la-
belled were added to a batch of 30 mM of L-lysine, HCl (Fluka, Buchs). The Boc-functional group was introduced into the amino acid via the azide by means of a pH-stat method.

After removing the completed peptide from the carrier-resin (2 % Styrol-DVP copolymer, Serva, Heidelberg) with TFA-HBr, the crude peptide preparation was purified on columns charged with Sephadex G-10 (Pharmacia, Uppsala) and ion exchange cellulose (Cellex CM, Bio Rad-Laboratories, Richmond, Calif.). The peptides were fractionated by means of NaCl-gradients and the fractions collected were monitored by flow spectrometers (PM 2D and PM 4, Zeiss, Oberkochen) at 2.200 nm. The purity of the peptides was checked by high voltage electrophoresis on paper using the apparatus of Hormuth, Heidelberg. After completion of purification the peptides were freeze-dried and stored in the refrigerator.

DNA-preparation

DNA from calf thymus and Micrococcus lysodeicticus was used for the binding studies. The calf thymus DNA was obtained from Worthington Biochem. Corp., Freehold, N.Y. and Sigma Chemicals Co., St. Louis, Mo. Residual protein was removed by phenol treatment and precipitation of the DNA with ethanol and isopropanol. The material was then dialyzed against a phosphate buffer (6 mM Na₂HPO₄, 2 mM NaH₂PO₄, 1 mM Na₂EDTA and 0.18 mM NaCl). Under these conditions, the calf thymus DNA could be stored at 4 °C for several months.

Micrococcus lysodeicticus DNA was isolated from frozen cell preparations supplied by Merck, Darmstadt (strain M 1108/T 4784) and from dried cell preparations purchased from Serva, Heidelberg. The isolation procedure was essentially the same as described by Marmur. The cells were suspended in phosphate buffer and agitated for 20 min. at 45 °C with lysozyme. Then the cells were broken up either over night with Pronase E (Merck, Darmstadt), or within 30 - 40 min. with Proteinase K (Merck, Darmstadt), while shaking at 31 °C.

When the viscous solution had become clear, 1,2 M sodium perchlorate was added. Finally the solution was extracted twice with chloroform: isoamylalcohol (25:1). The crude DNA was then precipitated with 96 % ethanol.

In order to remove RNA and residual proteins, two procedures were applied: a) Degradation of RNA by Ribonuclease A in connection with phenol extraction
of the residual proteins.
b) Purification of the DNA from RNA and residual proteins by isopycnic centrifu-
gation in a cesium chloride gradient<sup>22,23</sup>.

After purification the hyperchromicity for CT- and ML-DNA was 27% and 24.5% respectively. The T<sub>M</sub> values were 100°C and 87.5°C corresponding to 72 mole percent G-C for ML-DNA and 42 mole percent G-C for CT-DNA<sup>38</sup>.

**Equilibrium dialysis**

The equilibrium dialysis experiments were performed as described in detail by Wagner and Arav<sup>24</sup>. Immediately before starting dialysis experiments, the batches of DNA used were filtered and dialyzed for 12 hours against a 10 millimolar EDTA solution (pH 7.5), 6 hours against a 1 molar NaCl solution (pH 7.5) and five times 3 hours against a 0.02 molar NaCl solution (pH 6.0).

The equilibrium dialysis experiments were performed in 0.02 M NaCl (pH 6.0) with solutions containing 2.5 µmol of DNA per ml. The peptide stock solution contained 2 - 3 µmol peptide per ml. All salt solutions were saturated with chloroform to prevent bacterial growth. In order to exclude uncontrolled competition with other ions only Na<sup>+</sup> and Cl<sup>-</sup> ions were allowed to be in the system. As checked by a glass electrode the pH remained stable over the duration of the experiment.

When using a salt solution of relatively low ionic strength (0.02 M) one has to consider the possible influence of the Donnan effect. The Donnan effect was calculated according to Wagner and Arav<sup>24</sup> using the value of 0.36 for the effective charge of the phosphate group<sup>25</sup>. No influence on the K-values was found and only an average deviation of -3% for n. Since this deviation was within the experimental error of the equilibrium method, no correction was made for it.

The concentration of peptide in the stock solution was determined by measuring the nitrogen content with a microvolumetric method (Mikro Rapid N apparatus, Heraeus, Hanau). The DNA concentration was estimated spectrophotometrically at 260 nm. The molar extinction coefficients used for CT-DNA and ML-DNA were 6412 and 6923, respectively.
During the experiment the dialysis cells were rotated in a water bath of constant temperature (20 °C) until equilibrium was achieved after 36 - 48 hours. When the experiment was stopped 0.2 ml samples of the peptide solution of the chamber 2 (free peptide) were mixed with 10 ml of a toluene based scintillation fluor containing 33 % ethanol. The samples were counted in a Nuclear Chicago liquid scintillation spectrometer. By counting samples from chamber 1, it could be demonstrated that absorption of radioactive material on the walls of the dialysis cell and on the membrane was negligible.

The measurement of radioactivity obtained from dialysis experiments could be used, without further transformations, to compute peptide concentrations at the beginning and at the end of the experiment. The data obtained were treated according to Scatchard.

In general, straight lines could be drawn through the data by linear regression analysis. Deviations from straight lines were only observed when precipitation of the DNA-peptide complex occurred. The critical peptide/DNA ratio depended on the peptide used.

RESULTS

A number of experiments were carried out on the influence of DNA preparation techniques on the apparent binding constant using ML-DNA and Lys-Ala-Lys as ligand. It was found that ML-DNA purified by isopycnic centrifugation yielded somewhat higher binding constants than phenol extracted ML-DNA. Differences between high molecular and sonicated DNA could not be detected. To avoid differences due to preparative techniques, only high molecular CT-DNA purified by phenol extraction was used for further experiments. To check if the base composition of the CT- and ML-DNA affected the binding constants, binding experiments between Lys-Ala-Lys, Lys-Tyr-Lys, and Lys-Lys on the one hand and ML- and CT-DNA on the other were carried out. No significant variation in binding constants could be found.

All of the binding constants and n-values obtained from the experiments with CT-DNA and various peptides are listed in Table 1. Some representative Scatchard plots (Lys-Lys, Arg-Arg, and Lys-Tyr-Lys) are given in Fig. 1. Fig. 1 shows that the Scatchard plot yielded straight lines.
Table 1. List of the binding constants (M^{-1}) and n-values (a number of
model peptides per DNA molecule group), obtained by absorbance changes
in 0.02 M NaCl solution, pH 6.0.

<table>
<thead>
<tr>
<th>Peptide</th>
<th>K (M^{-1})</th>
<th>n</th>
<th>( K_{d} ) (M)</th>
<th>( K_d ) (M)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Arg-Lys</td>
<td>5.2 × 10^{-5}</td>
<td>0.22</td>
<td>2.12 ± 0.14</td>
<td>1.34 ± 0.27</td>
</tr>
<tr>
<td>Lys-Lys</td>
<td>3.2 × 10^{-5}</td>
<td>0.32</td>
<td>2.23 ± 0.33</td>
<td>1.53 ± 0.25</td>
</tr>
<tr>
<td>Lys-Lys-Lys</td>
<td>2.36 ± 0.34</td>
<td>0.36</td>
<td>2.45 ± 0.45</td>
<td>1.72 ± 0.31</td>
</tr>
<tr>
<td>Lys-Lys-Lys-Lys</td>
<td>2.29 ± 0.29</td>
<td>0.39</td>
<td>2.38 ± 0.32</td>
<td>1.64 ± 0.25</td>
</tr>
<tr>
<td>Lys-Lys-Lys-Lys</td>
<td>3.29 ± 0.23</td>
<td>0.30</td>
<td>2.43 ± 0.34</td>
<td>1.76 ± 0.28</td>
</tr>
<tr>
<td>Lys-Lys-Lys-Lys</td>
<td>2.30 ± 0.32</td>
<td>0.35</td>
<td>2.30 ± 0.28</td>
<td>1.69 ± 0.21</td>
</tr>
<tr>
<td>Lys-Lys-Lys-Lys</td>
<td>2.29 ± 0.34</td>
<td>0.36</td>
<td>2.38 ± 0.32</td>
<td>1.64 ± 0.25</td>
</tr>
<tr>
<td>Lys-Lys-Lys-Lys</td>
<td>2.30 ± 0.28</td>
<td>0.35</td>
<td>2.30 ± 0.28</td>
<td>1.69 ± 0.21</td>
</tr>
</tbody>
</table>

Figure 1: Scatchard plots for the binding of some model peptides to CT-DNA, measured in 0.02 M NaCl solution, pH 6.0.
The results presented in Table 1 allow the following interpretations:

1. By comparing the binding constants of dilysine, Lys-Ala-Lys, Lys-(Ala)$_2$-Lys and Lys-(Ala)$_3$-Lys, which are practically the same (K = 3.0 mM$^{-1}$), it can be concluded that the distance between the c-NH$_3^+$-groups of the terminating lysine residues has no influence on the binding strength of these peptides to DNA.

2. A similar experiment with dilysine, Lys-Gly-Lys, and Lys-(Gly)$_2$-Lys as ligands yielded different results. There is a slight increase in the binding constant with each added glycine residue as compared with dilysine.

3. The observation that glycine within the peptide structure might enhance the binding of such a peptide to DNA is further supported by binding studies between DNA and Lys-(Ala)$_2$-Lys, Lys-Gly-Ala-Lys, and Lys-(Gly)$_2$-Lys. In this series of peptides alanine is successively replaced by glycine and, correspondingly, the binding constants from Lys-(Ala)$_2$-Lys (K = 3.4 mM$^{-1}$) to Lys-(Gly)$_2$-Lys (K = 3.64 mM$^{-1}$) increase. The binding constant of the transition peptide Lys-Gly-Ala-Lys (K = 3.25 mM$^{-1}$) lies between those of Lys-(Gly)$_2$-Lys and Lys-(Ala)$_2$-Lys, as might be expected.

4. Beside peptides containing alanine and glycine we also prepared a peptide containing leucine, (Lys-Leu-Lys). As compared to Lys-Ala-Lys and Lys-Gly-Lys it shows less binding strength.

5. The comparison of Lys-Tyr-Lys with Lys-Ala-Lys and Lys-Gly-Lys reveals that the aromatic function of tyrosine enhances the binding strength significantly.

6. Comparing the binding constants of dilysine (K = 2.85 mM$^{-1}$) and diarginine (K = 8.89 mM$^{-1}$) it becomes evident that diarginine binds much stronger to DNA than dilysine.

7. The affinity constants of dilysine (K = 2.86 mM$^{-1}$) and trilysine (K = 39.6 mM$^{-1}$) show that the addition of one c-NH$_3^+$-group to the dipeptide increases the binding strength drastically.
8. The values for $n$ ranging from 0.22 to 0.32 indicate 3 to 4 binding sites on the DNA per peptide molecule.

9. It is possible to arrange the lysine peptides with the different intermediary amino acid residues in a series with increasing binding constants

leucine < alanine < glycine < tyrosine.

DISCUSSION

In recent years many investigations on the binding of amino acids or small peptides to DNA have placed special emphasis on the interaction of aromatic amino acid residues to the DNA backbone\(^{3,10,14,30}\).

In addition, there have been also attempts to shed some light on the influence of binding forces which do not arise from aromatic amino acid residues using other detection methods, for example viscometry, UV, and CD spectroscopy, establishment of melting profiles and binding studies by means of equilibrium techniques\(^{13,14,15,16,31,32}\).

Before discussing the results it seems necessary to comment on the Scatchard representation. On theoretical grounds Scatchard plots of binding data obtained from experiments with ligands covering more than one lattice residue on the DNA, should result in non-linear binding curves\(^{33,34}\). Since under our experimental conditions straight lines were obtained even with the highest $r$-values used, the classical Scatchard representation was still adequate. This is also valid for the evaluation of $n$. Obviously the actual curvature for our ligands, covering an average of 3 binding sites is still within experimental error.

The paramount importance of the $\epsilon-NH_3^+$-groups of lysine peptides for the binding to polynucleotide structures has already been demonstrated by Latt and Sober\(^{31}\). This fact is supported by our findings and the results of Gabbay et al.\(^{15}\), who also found that the binding strength between lysine peptides and DNA increases drastically with the number of lysine residues added to the peptide structure.

From the $n$-values (Table 1) it can be concluded that peptides containing
two lysine residues occupy about 3 phosphates on the DNA, those with 3 lysine residues (trilysine) about four. This is in agreement with the results of Brun et al. and Durand et al., who did binding studies with poly A and lysine peptides of the same type we used (Lys-X-Lys), applying fluorescence and CD techniques, respectively. From the pH and ionic strength dependence of their fluorescence data they concluded that the model peptide Lys-Tyr-Lys, covering 3 to 4 $P$O$_4$ groups on the DNA, provides only two electrostatic bonds (the $\alpha$-$NH_3^+$-group and one $\epsilon$-$NH_3^+$-group) for binding to DNA.

Considering our experimental conditions (pH 6.0), there is no doubt that the $\alpha$-amino group of our model peptides is completely protonated and thus is likely to be involved in bond formation. However, binding data do not permit any conclusion on how many electrostatic bonds participate and whether or not the $\alpha$-amino-group is involved in binding. Since poly A and DNA differ as regards to their secondary structures drawing analogies concerning bond formation may not be feasible.

As already mentioned diarginine binds much stronger to DNA than dilysine. This is in accordance with respective concepts and other experimental evidence. Most of the investigations dealing with the weak non ionic interactions directed their special concern to the aromatic amino acids. From the work of Sellini et al., Dimicoli and Hélène and Brun et al. it seems quite clear that in a peptide like Lys-Tyr-Lys electrostatic interactions, stacking of the aromatic ring with nucleic acid bases and hydrogen bonding contribute to the overall binding of this molecule to DNA. The affinity constants found for Lys-Tyr-Lys (5.14 mM$^{-1}$) and Lys-Lys (2.85 mM$^{-1}$) show that effects like those mentioned above are superimposed upon the electrostatic interactions.

In the literature very little data could be found concerning the participation of the non aromatic amino acids in the binding to DNA. Gabbay et al. have presented extensive lists of binding constants of di- and tri-peptide amides but from their data one can conclude only that the amino acid sequence of the peptide has some influence on the binding strength. The experiments in this paper indicate that glycine within the peptide chain does enhance the binding strength as compared to alanine and leucine. This is probably due to shielding of the peptide bond by the side chain of ala-
nine and leucine which does not exist with the glycine residue. This is supported by results of Arfman et al.\(^\text{37}\). Their binding studies in which 5' GMP and lysine copolyampholytes with different amino acids residue incorporated were used, revealed that the highest binding constants were for the lysine-glycine copolymer. The melting profiles established by Novak and Dohnal\(^\text{32}\) with complexes of DNA and tyrosine-glycine peptides of varying glycine content also give some hint that glycine is involved in binding to DNA.

Taking into consideration the results of all the binding studies discussed, it can be concluded that the binding strength of lysine oligopeptides is significantly modified by intermediary neutral or aromatic amino acid residues. It seems that the more or less hydrophobic character of the spacer amino acid side chain is more important to binding than the varying chain length.

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