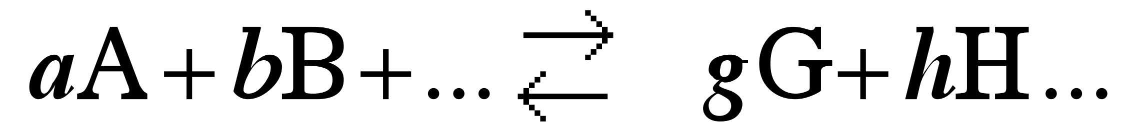
Binding of ligands to a macromolecule

- **General description of ligand binding**
  - –the esssentials
  - –thermodynamics
  - –Adair equation
- **Simple equilibrium binding**
  - –stoichiometric titration
  - –equilibrium binding/dissociation constant
- **Complex equilibrium binding**
  - –cooperativity
  - –Scatchard plot and Hill Plot
  - –MWC and KNF model for cooperative binding

ΔG of an reaction in equilibrium



$$0 = \Delta G^0 + RT \ln\left(\frac{[\mathrm{G}]^{\mathrm{g}}[\mathrm{H}]^{\mathrm{h}} \ldots}{[\mathrm{A}]^{\mathrm{a}}[\mathrm{B}]^{\mathrm{b}} \ldots}\right)_{\mathrm{Eq}}$$

$$\Delta G^0 = -RT \ln\left(\frac{[\mathrm{G}]^{\mathrm{g}}[\mathrm{H}]^{\mathrm{h}} \ldots}{[\mathrm{A}]^{\mathrm{a}}[\mathrm{B}]^{\mathrm{b}} \ldots}\right)_{\mathrm{Eq}} = -RT \ln K$$

$$K = \left(\frac{[\mathrm{G}]^{\mathrm{g}}[\mathrm{H}]^{\mathrm{h}} \ldots}{[\mathrm{A}]^{\mathrm{a}}[\mathrm{B}]^{\mathrm{b}} \ldots}\right)_{\mathrm{Eq}} = \exp\left(\frac{-\Delta G^0}{RT}\right)$$

The mass equation law for binding of a protein P to its DNA D

$$D_{\text{free}} + P_{\text{free}} \rightleftarrows DP \qquad K_1 = \frac{D_{\text{free}} \cdot P_{\text{free}}}{DP}$$

binding of the first proteins with the dissociation constant K_1

D_{free}, concentration free DNA; P_{free}, concentration free protein

$$\text{binding constant } K_{\text{B}} = \frac{1}{\text{dissociation constant } K_{\text{D}}}$$

What is the meaning of the dissociation constant for binding of a single ligand to its site?

1. K_D is a concentration and has units of mol per liter

2. K_D gives the concentration of ligand that saturates 50% of the sites (when the total sit concentration ismuch lower than K_D)

3. Almost all binding sites are saturated if the ligand concentration is 10 x K_D

4. The dissociatin constant K_D is related to Gibbs free energy ΔG by the relation $\Delta G = - R\, T \ln(K_D)$

Increasing complexity of binding

all binding sites are
equivalent and independent

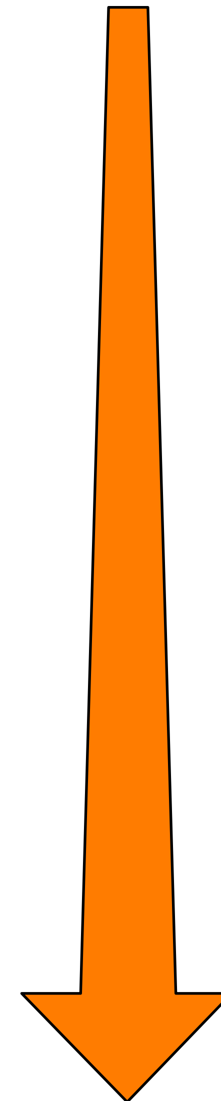
cooperativity ↙ ↘ ***heterogeneity***

all binding sites are
equivalent and not independent

all binding sites are
independent but not equivalent

heterogeneity ↘ ↙ ***cooperativity***

all binding sites are
not equivalent and not independent

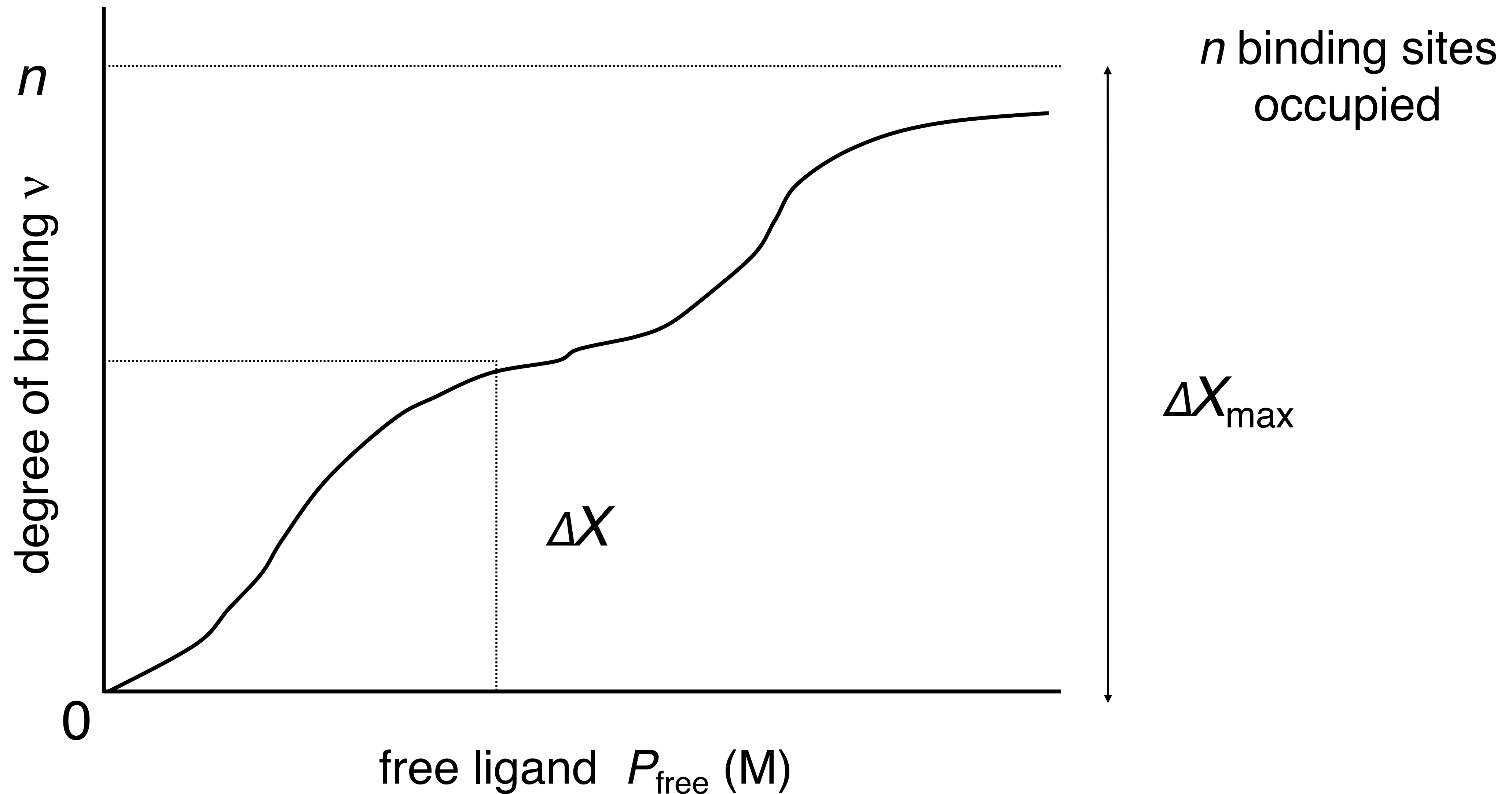


simple

difficult

very difficult

Titration of a macromolecule *D* with *n* binding sites for the ligand *P* which is added to the solution



$$\frac{\Delta X}{\Delta X_{max}} = \frac{\nu}{n} = \theta \text{ (fraction saturation)} \qquad \nu = \frac{[\text{bound ligand } P]}{[\text{macromolecule } D]}$$

Schematic view of gel electrophoresis to analyze protein-DNA complexes

Mark M. Garner and Arnold Revzin

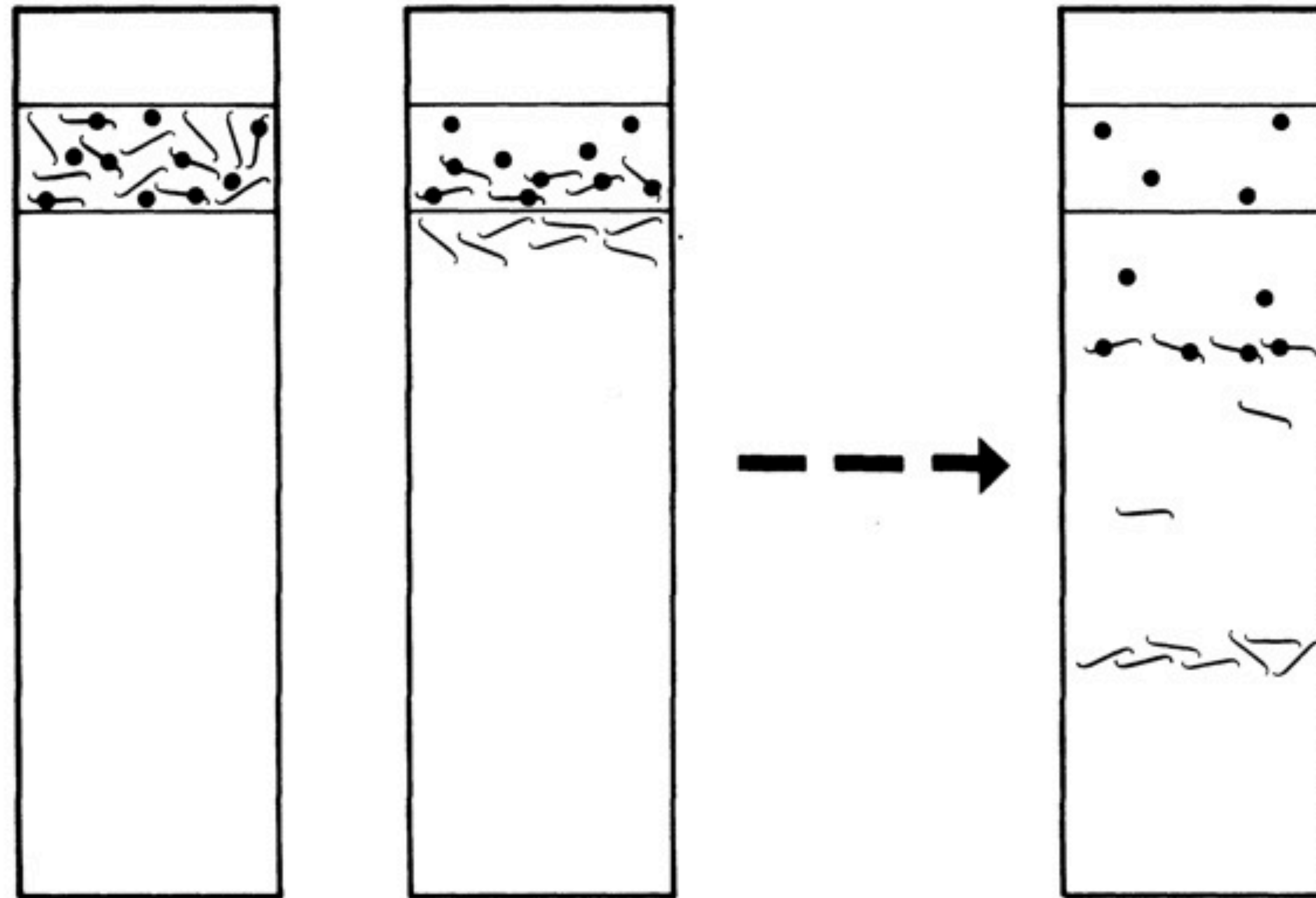
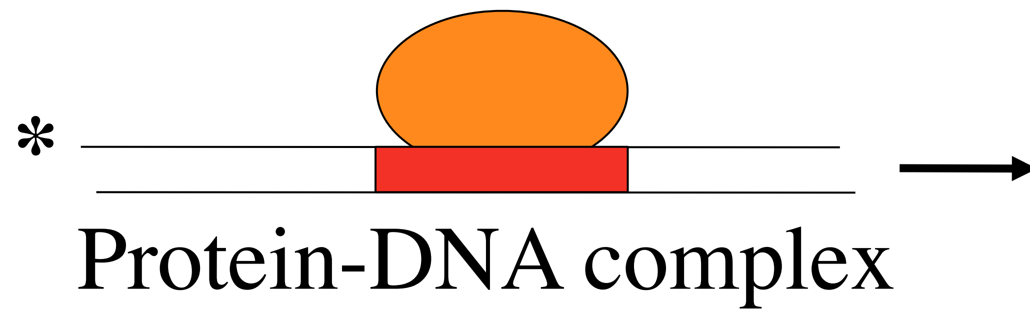


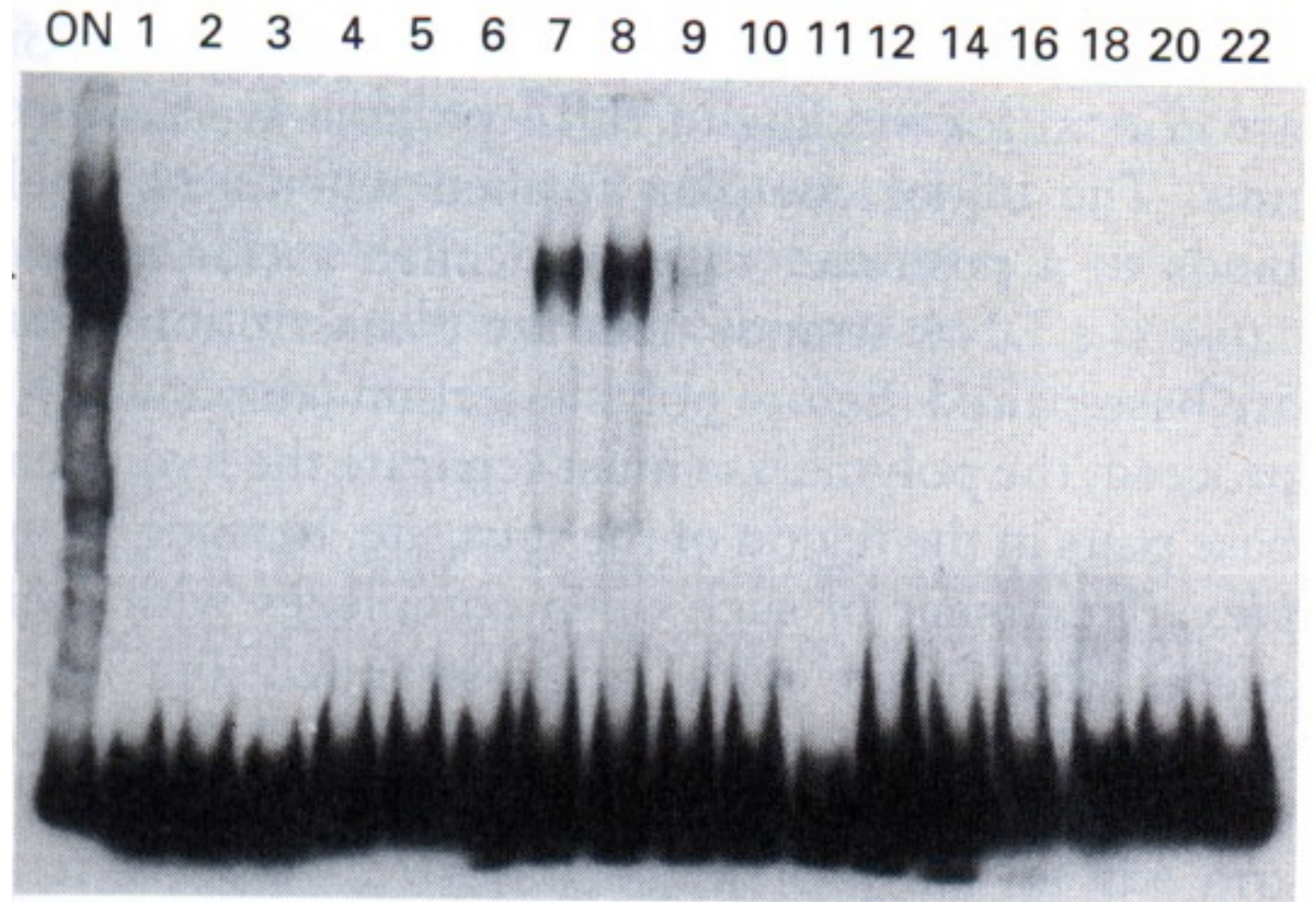
Figure 1. Schematic diagram of the gel retardation method. The filled circles represent protein, curved lines represent linear DNA fragments. The left-hand panel illustrates the DNA – protein solution loaded on to the gel. The middle panel shows free DNA entering the gel just after the power is turned on. The right-hand panel depicts the situation later in the run; bands of complexes and of free DNA are seen. If the complexes dissociate during electrophoresis, the DNA released never catches up with the main band of free DNA.

"Gel shift": electorphoretic mobility shift assay ("EMSA") for DNA-binding proteins



Protein-DNA complex

Free DNA probe



1. Prepare labeled DNA probe
2. Bind protein
3. Native gel electrophoresis

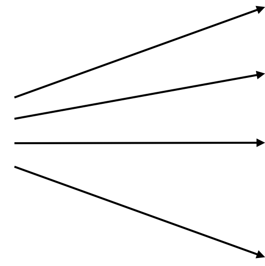
Advantage: sensitive, fmol DNA

Disadvantage: requires stable complex; little "structural" information about which protein is binding

EMSA of Lac repressor binding to operator DNA

From (a) to (j) the concentration
of lac repressor is increased.

Complexes with



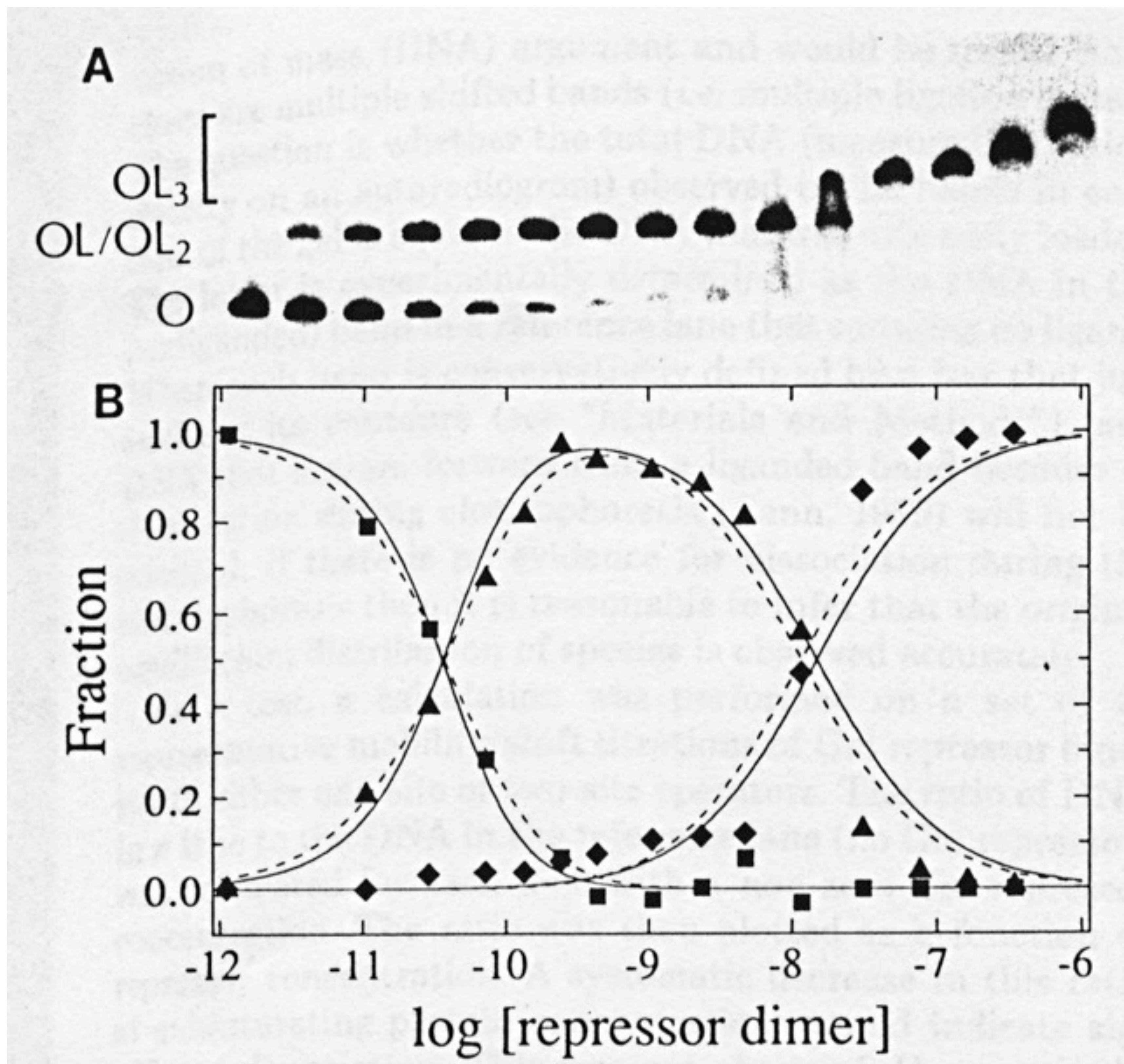
Free DNA

a b c d e f g h i j

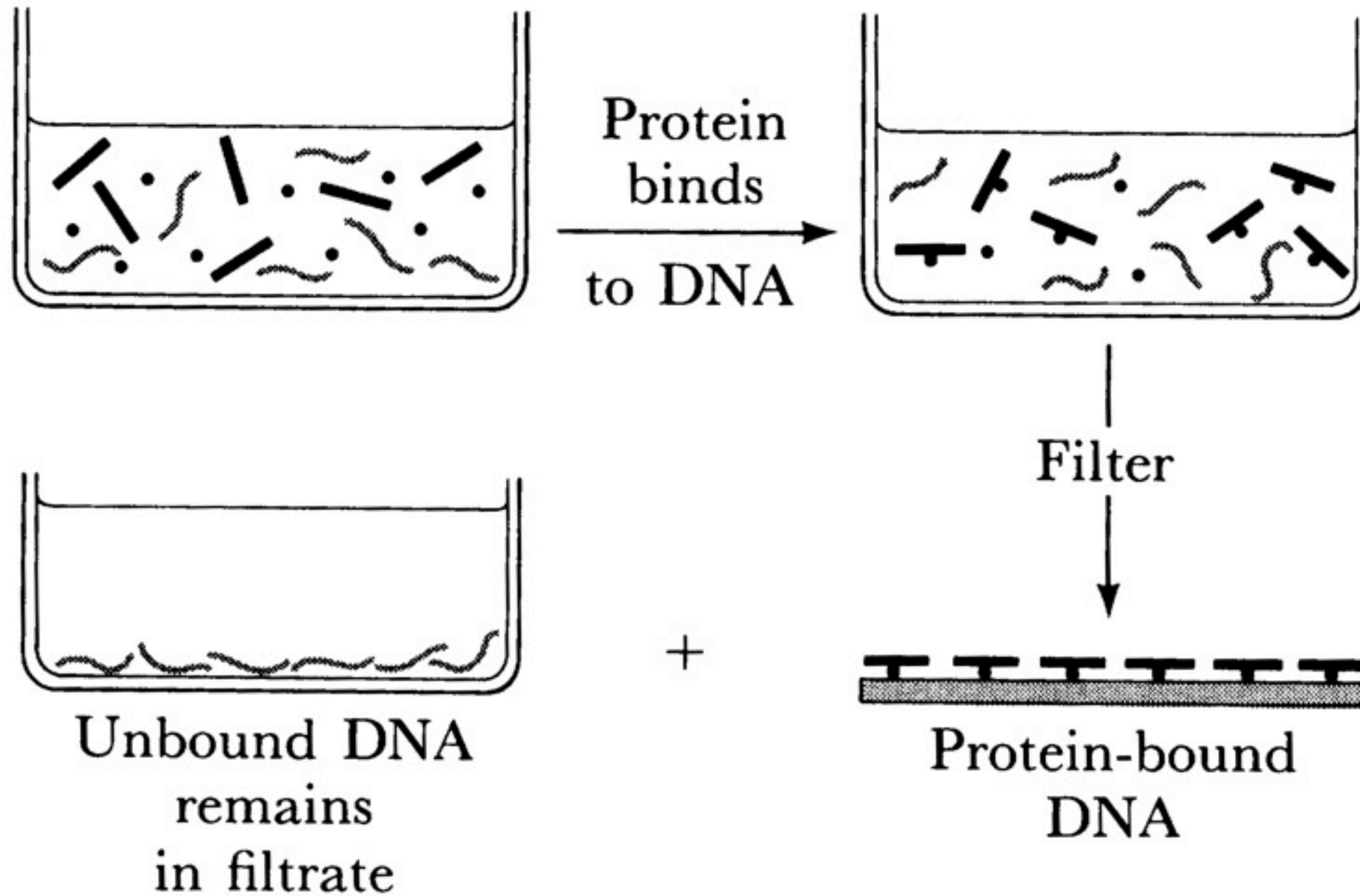
4
3
2
1
F

Figure 1. Titration of the 203 bp L8-UV5 lactose promoter-operator fragment with *lac* repressor. The DNA fragment concentration was 18.5 nM *Lac* repressor concentrations were: 0, 12.4, 24.8, 37.0, 49.4, 61.6, 74.0, 98.8, 123.4 and 148.0 nM for samples (a)–(j) respectively. Samples were incubated for 30 min at room temperature in 10 mM Tris (pH 8.0 at 21 °C), 1 mM EDTA, 50 mM KCl, and applied to a 5 % polyacrylamide gel equilibrated with the same buffer. Electrophoresis was at 8 V/cm for 2 h. The repressor: fragment ratios [1] of some complexes are given of the left margin. Band F denotes free DNA.

Measuring binding constants for lambda repressor on a gel

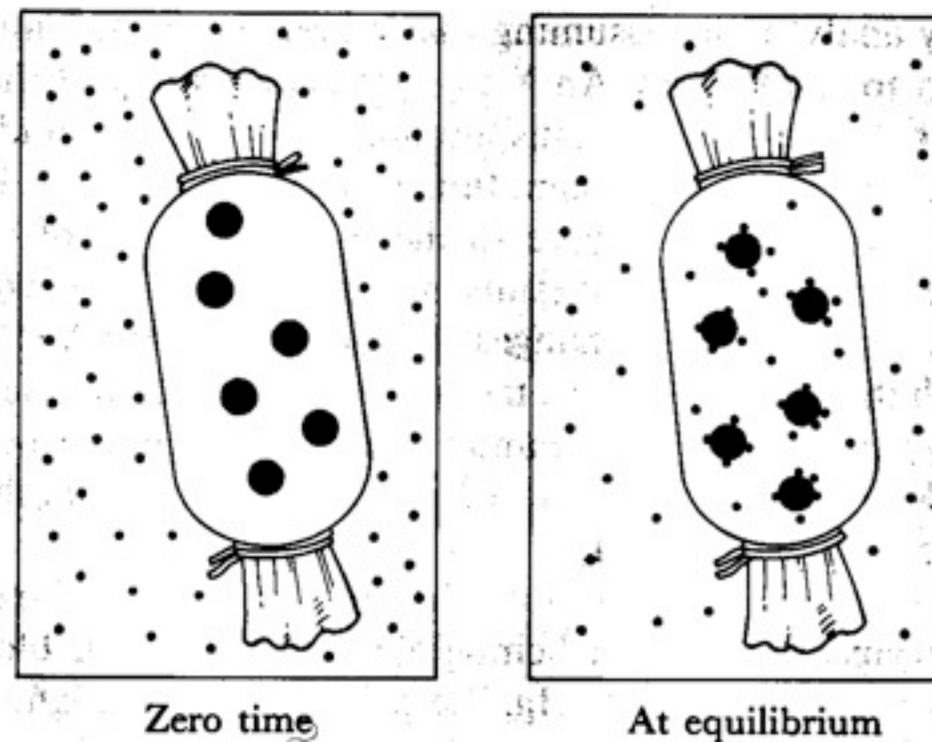


Principle of filter-binding assay



Binding measurments by equilibrium dialysis

A macromolecule is dialyzed against a solution of ligand. Upon reaching equilibrium, the ligand concentration is measured inside and outside the dialysis chamber. The excess ligand inside the chamber corresponds to bound ligand.



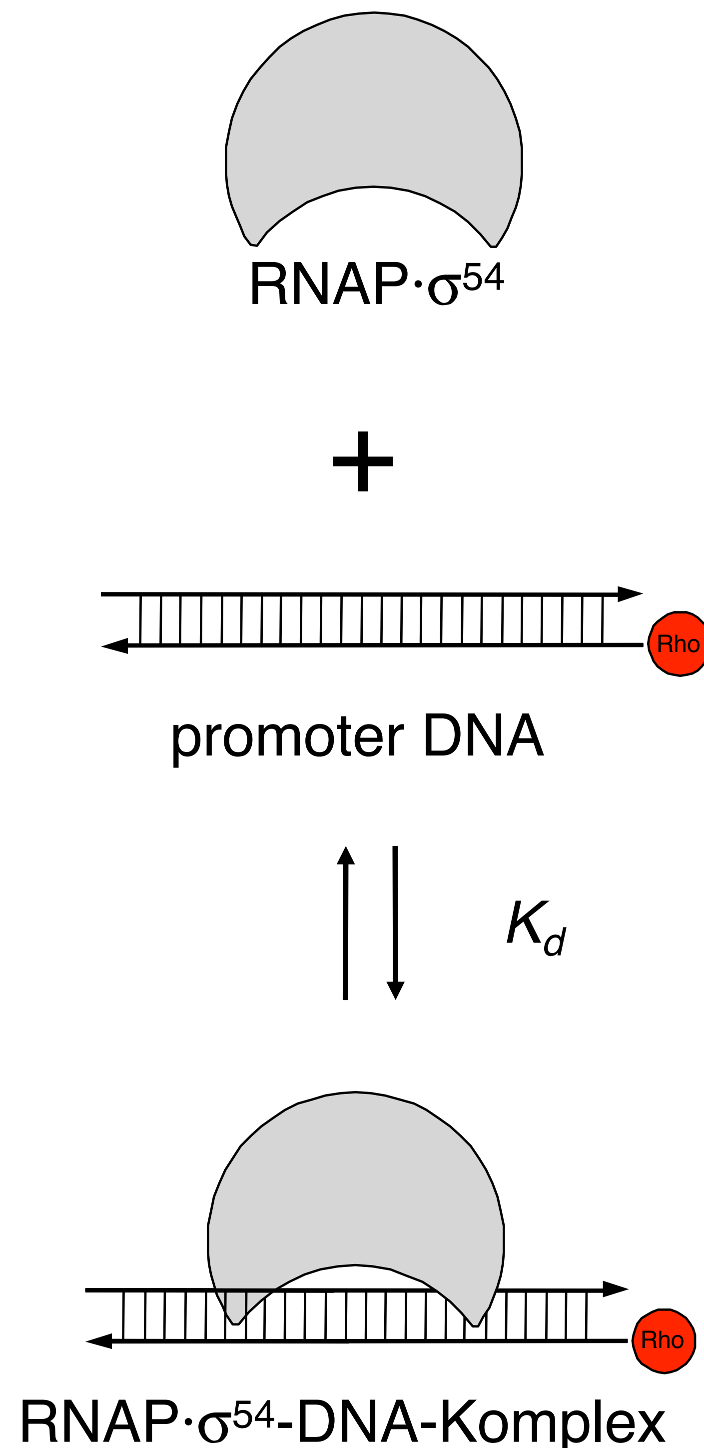
$$\nu = \frac{[X]_{in} - [X]_{out}}{M}$$

- direct measurement of binding

-non-specific binding will obscure results, work at moderate ionic strength (≥50 to avoid the *Donnan Effect* (electrostatic interactions between the macromolecule and a charged ligand.

- needs relatively large amounts of material

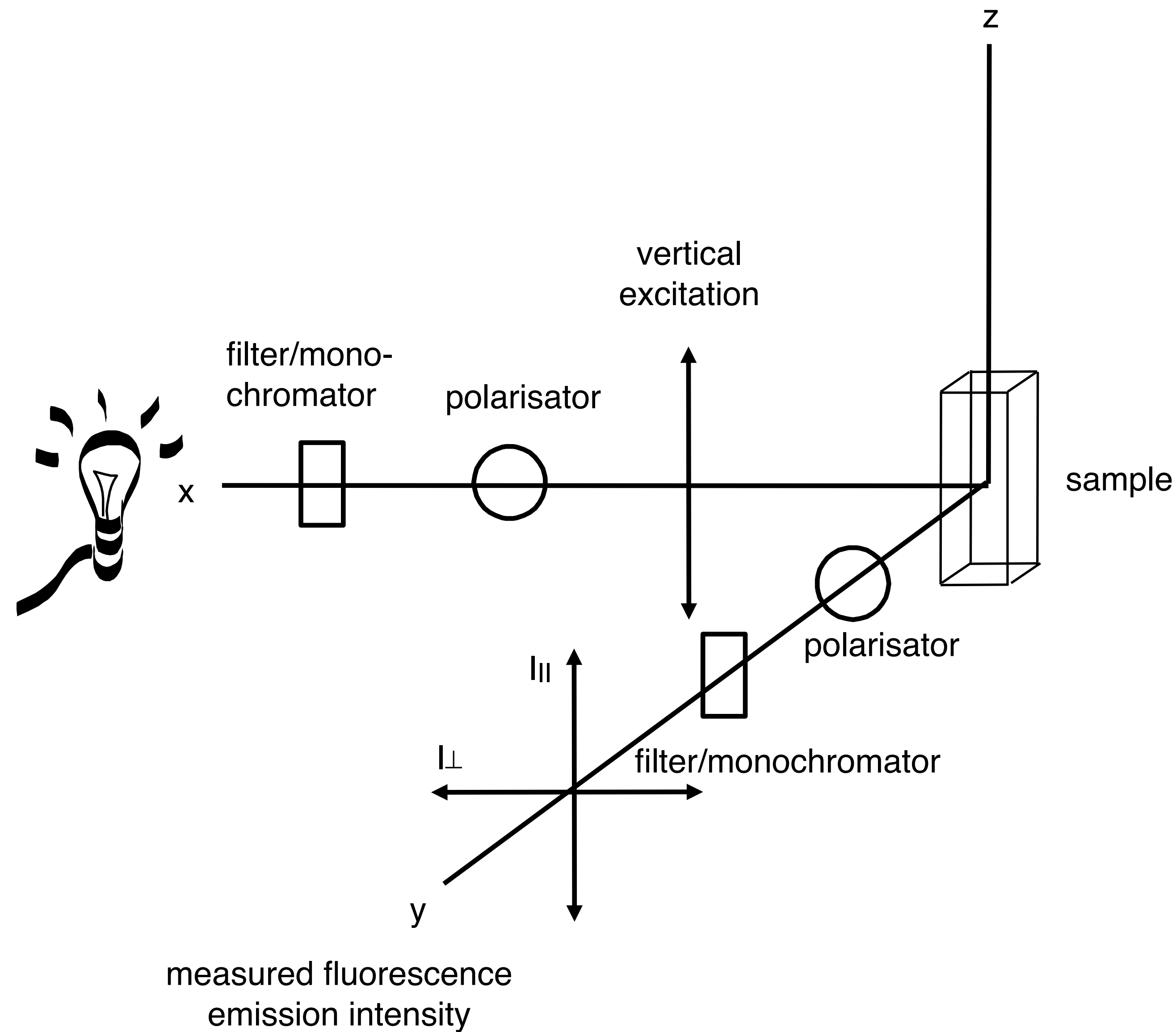
Analysis of binding of RNAP·σ^{54} to a promoter DNA sequence by measurements of fluorescence anisotropy



free DNA with a fluorophore
with high rotational diffusion
-> low fluorescence anisotropy r_{min}

RNAP-DNA complex
with low rotational diffusion
-> high fluorescence anisotropy r_{max}

How to measure binding of a protein to DNA? One possibility is to use fluorescence anisotropy

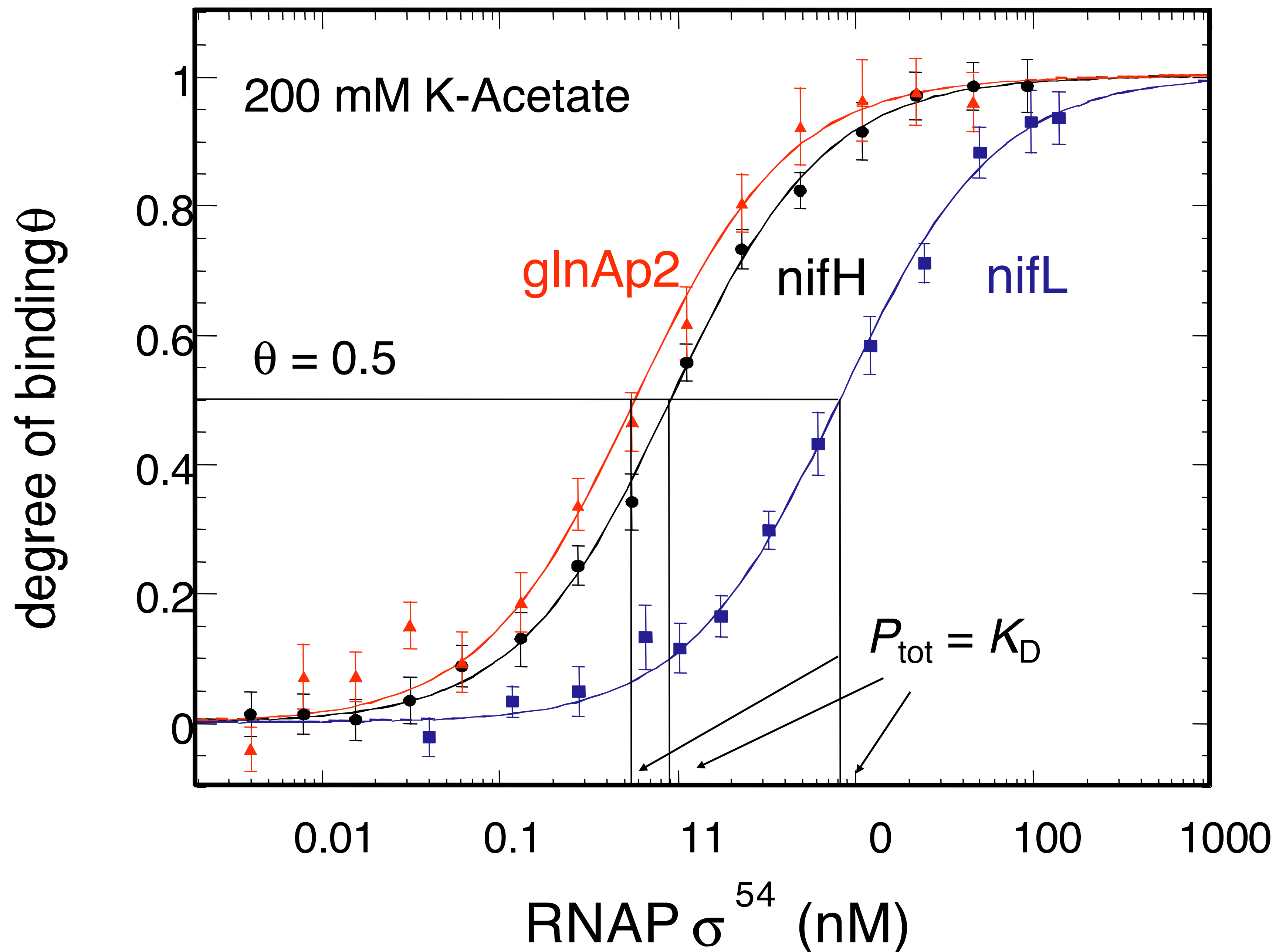


$$r = \frac{I_{II} - I_{\perp}}{I_{II} + 2I_{\perp}}$$

Definition of fluorescence anisotropy *r*

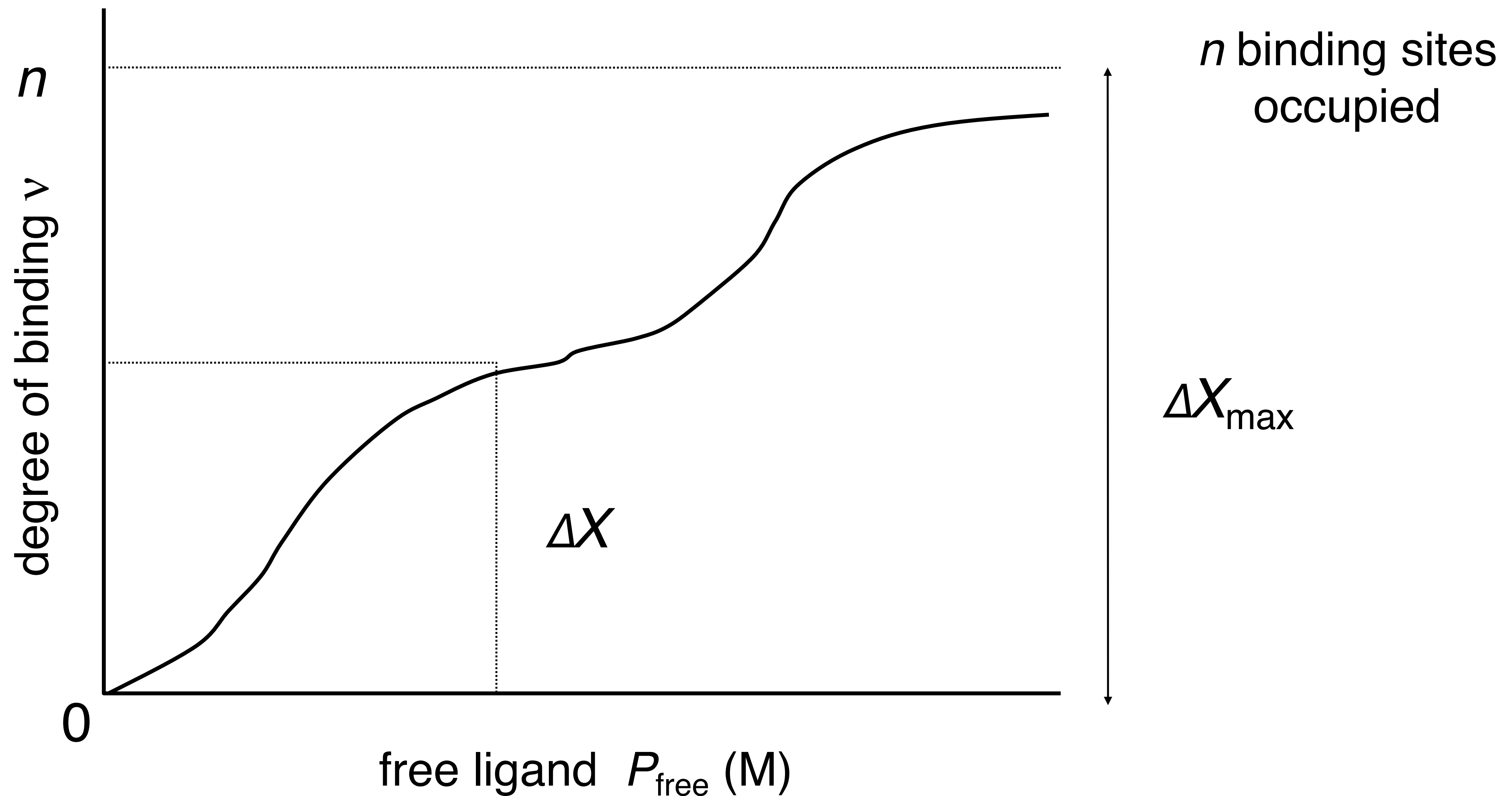
The anisotropy *r* reflects the rotational diffusion of a fluorescent species

Measurements of fluorescence anisotropy to monitor binding of RNAP·σ^{54} to different promoters



Vogel, S., Schulz A. & Rippe, K.

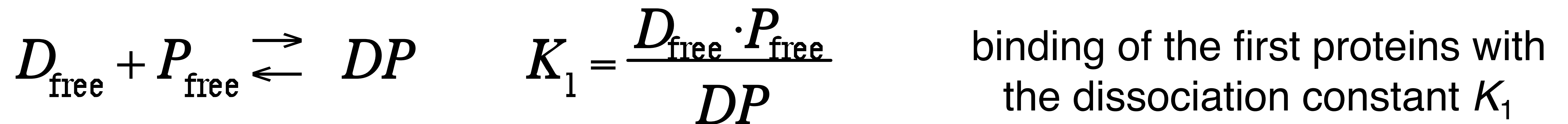
Titration of a macromolecule D with n binding sites for the ligand P which is added to the solution



$$\frac{\Delta X}{\Delta X_{max}} = \frac{\nu}{n} = \theta \ \text{(fraction saturation)}$$

$$\nu = \frac{[\text{bound ligand } P]}{[\text{macromolecule } D]}$$

Example: binding of a protein *P* to a DNA-fragment *D* with one or two binding sites



$$D_{\text{free}} + P_{\text{free}} \rightleftarrows DP \qquad K_1 = \frac{D_{\text{free}} \cdot P_{\text{free}}}{DP}$$

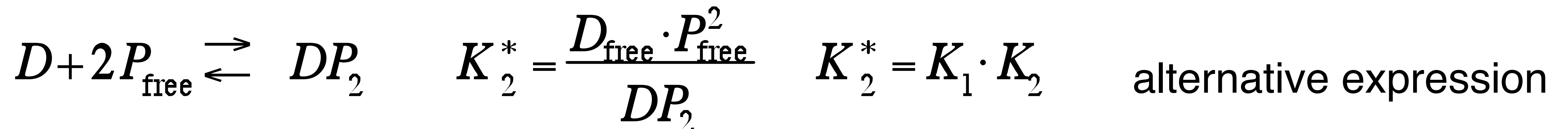
binding of the first proteins with the dissociation constant K_1

D_{free}, concentration free DNA; P_{free}, concentration free protein;

DP, complex with one protein; DP_2, complex with two proteins;

$$DP + P_{\text{free}} \rightleftarrows DP_2 \qquad K_2 = \frac{DP \cdot P_{\text{free}}}{DP_2}$$

binding of the second proteins with the dissociation constant K_2



$$D + 2P_{\text{free}} \rightleftarrows DP_2 \qquad K_2^* = \frac{D_{\text{free}} \cdot P_{\text{free}}^2}{DP_2} \qquad K_2^* = K_1 \cdot K_2$$

alternative expression

$$\text{binding constant } K_{\text{B}} = \frac{1}{\text{dissociation constant } K_{\text{D}}}$$

Definition of the degree of binding ν

$$\nu = \frac{[\text{bound ligand } P]}{[\text{macromolecule } D]}$$

degree of binding ν

$$\nu_1 = \frac{DP}{D_{\text{free}} + DP}$$

ν for one binding site

$$\nu_2 = \frac{DP + 2 \times DP_2}{D_{\text{free}} + DP + DP_2}$$

ν for two binding sites

$$\nu = \frac{\sum_{i=1}^{n} i \cdot \frac{1}{K_i} \cdot D_{\text{frei}} \cdot P_{\text{frei}}^{i}}{\sum_{i=0}^{n} \frac{1}{K_i} \cdot D_{\text{frei}} \cdot P_{\text{frei}}^{i}} = \frac{\sum_{i=1}^{n} i \cdot \frac{1}{K_i} \cdot P_{\text{frei}}^{i}}{\sum_{i=0}^{n} \frac{1}{K_i} \cdot P_{\text{frei}}^{i}} \quad \text{mit } K_0 = 1$$

ν for *n* binding sites (Adair equation)

Binding to a single binding site: Deriving an expression for the degree of binding ν or the fraction saturation θ

$$D_{\text{free}} + P_{\text{free}} \rightleftarrows DP \qquad K_D = \frac{D_{\text{free}} \cdot P_{\text{free}}}{DP}$$

from the Adair equation we obtain:

$$\nu_1 = \theta = \frac{\frac{1}{K_D} \cdot P_{\text{free}}}{1 + \frac{1}{K_D} \cdot P_{\text{free}}} \quad \Leftrightarrow \quad \nu_1 = \theta = \frac{P_{\text{free}}}{K_D + P_{\text{free}}}$$

Often the concentration P_{free} can not be determined but the total concentration of added protein P_{tot} is known.

$$P_{\text{free}} = P_{\text{tot}} - \nu_1 \cdot D_{\text{tot}}$$

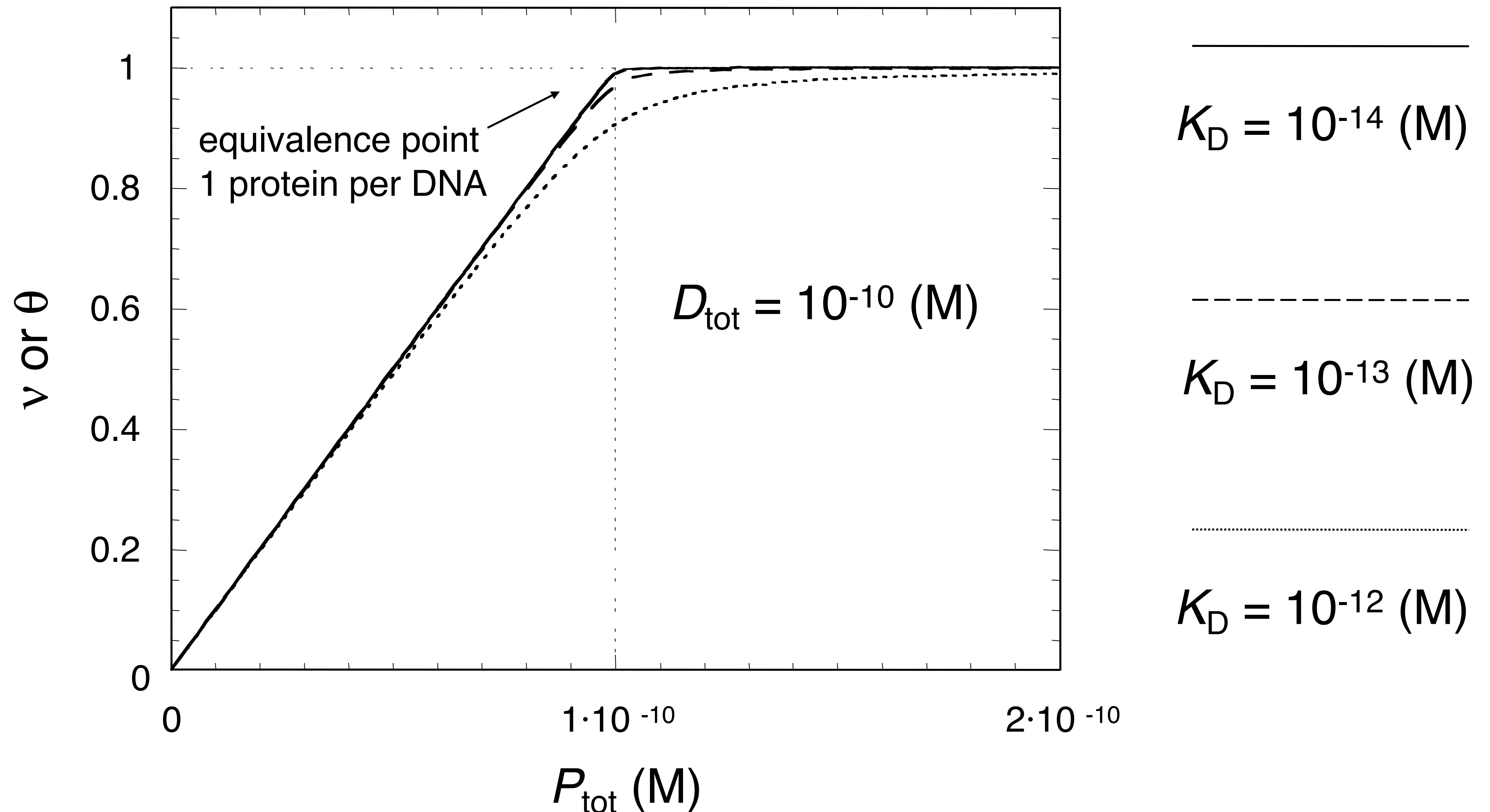
$$\nu_1 = \frac{D_{\text{tot}} + P_{\text{tot}} + K_D - \sqrt{\left(D_{\text{tot}} + P_{\text{tot}} + K_D\right)^2 - 4 \cdot D_{\text{tot}} \cdot P_{\text{tot}}}}{2 \cdot D_{\text{tot}}}$$

Stoichiometric titration to determine the number of binding sites

$$\frac{\nu}{n} = \theta$$

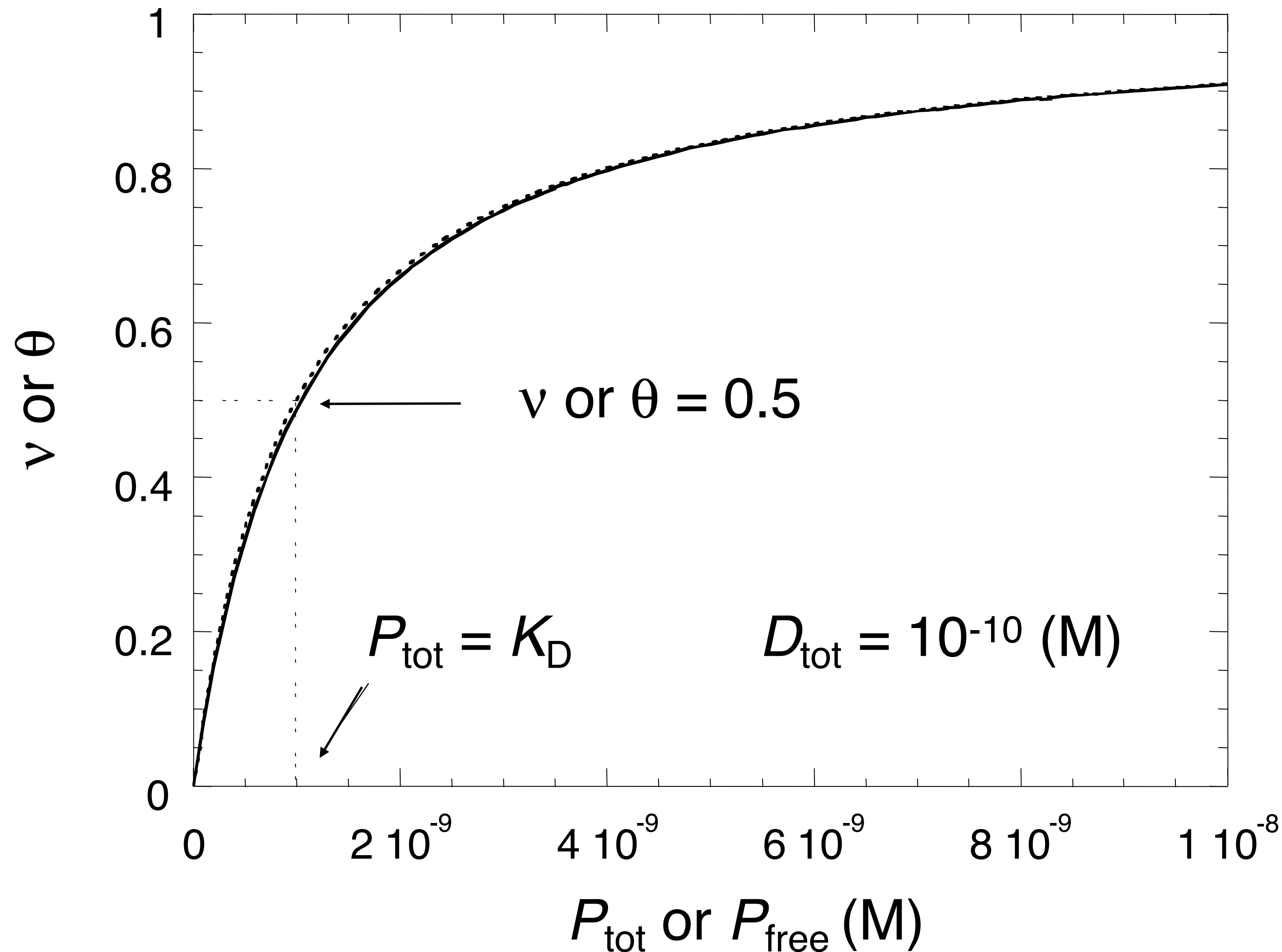
$$\text{for } n = 1$$

$$\nu = \theta$$



To a solution of DNA strands with a single binding site small amounts of protein P are added. Since the binding affinity of the protein is high (low K_D value as compared to the total DNA concentration) practically every protein binds as long as there are free binding sites on the DNA. This is termed “stoichiometric binding” or a “stoichiometric titration”.

Binding to a single binding site. Titration of DNA with a protein for the determination of the dissociation constant K_D



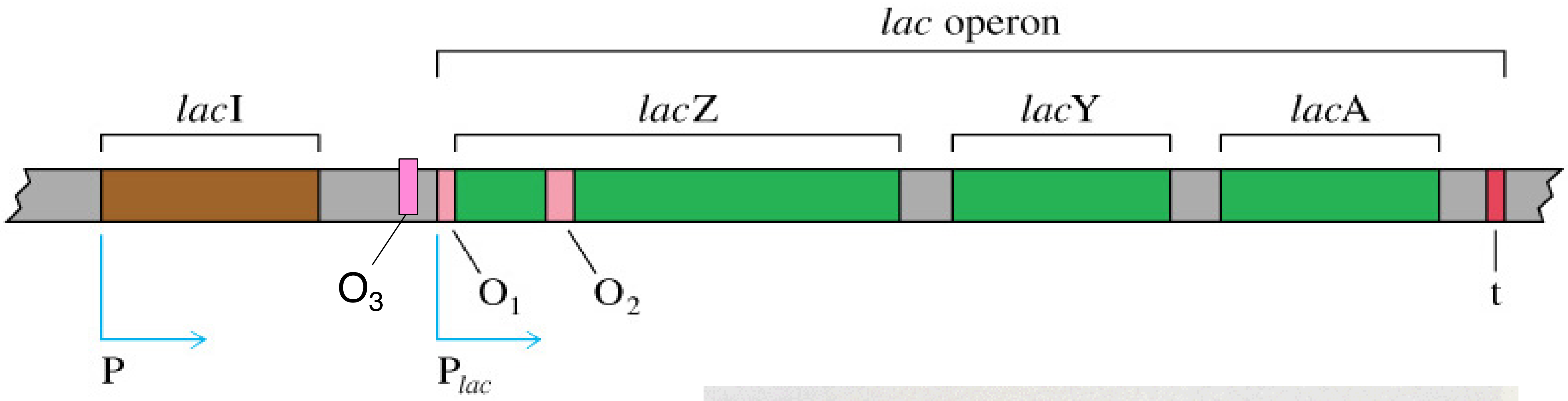
$K_D = 10^{-9}$ (M) $\quad \nu_1 = \frac{P_{free}}{P_{free} + K_D}$

$K_D = 10^{-9}$ (M) $\quad \nu_1 = \frac{P_{tot}}{P_{tot} + K_D}$

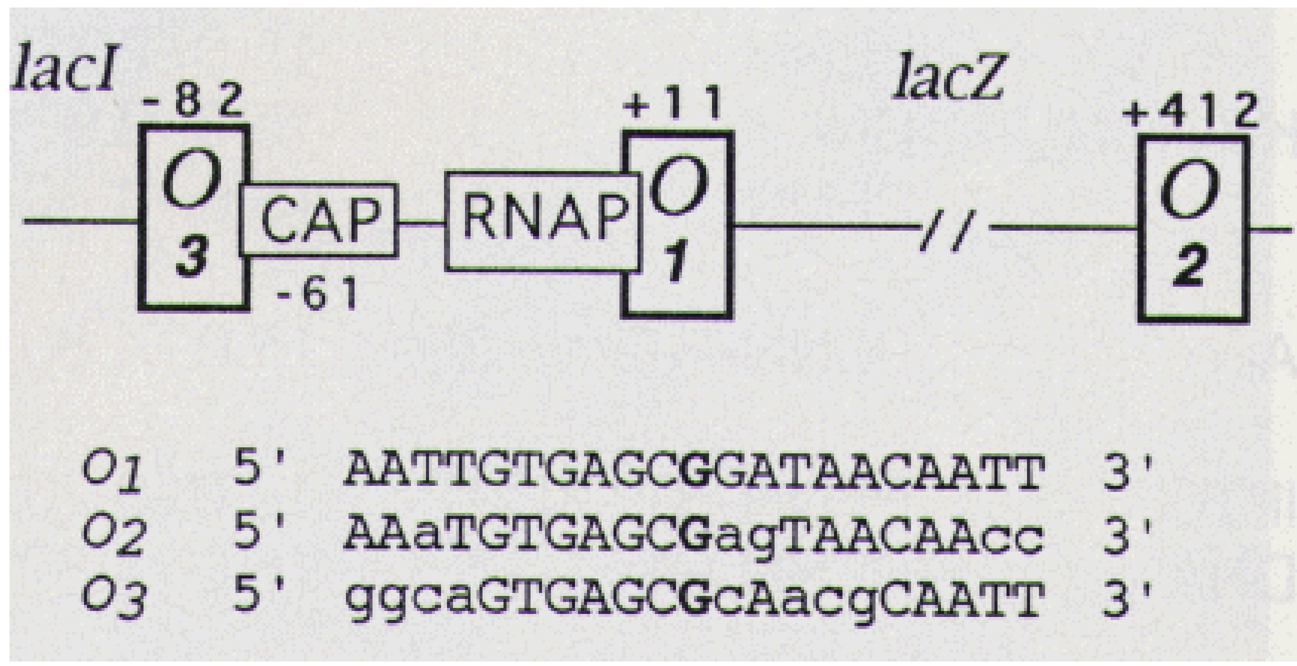
$$\nu_1 = \frac{P_{free}}{P_{free} + K_D} \approx \frac{P_{tot}}{P_{tot} + K_D} \quad \text{if } P_{free} \approx P_{tot} \quad \text{d. h. } 10 \times D_{tot} \le K_D$$

Lac repressor binding to DNA
-
linking structure and thermodynamics

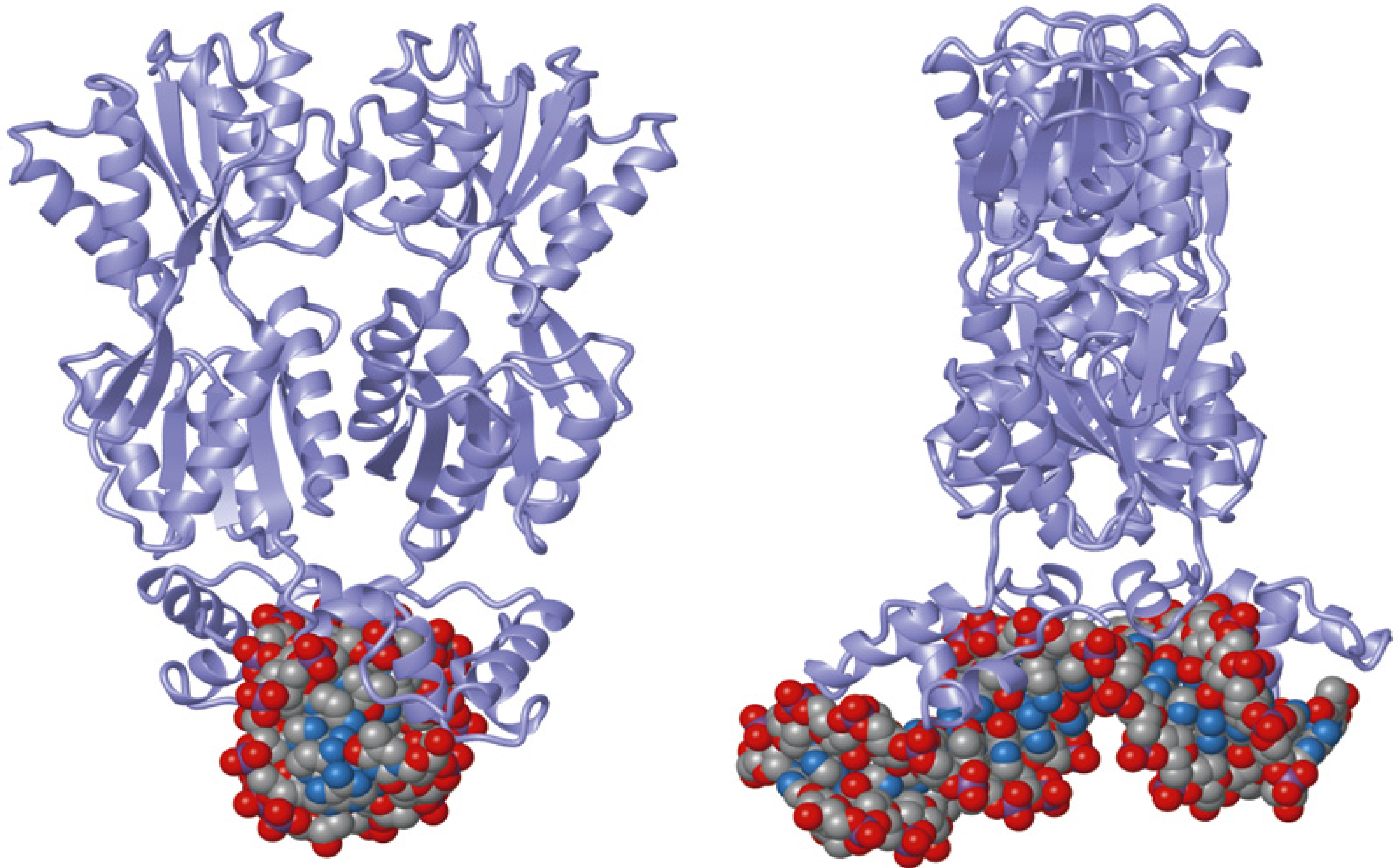
Organization of the genes regulated by Lac repressor, a transcription repressor protein in the bacterium E. coli



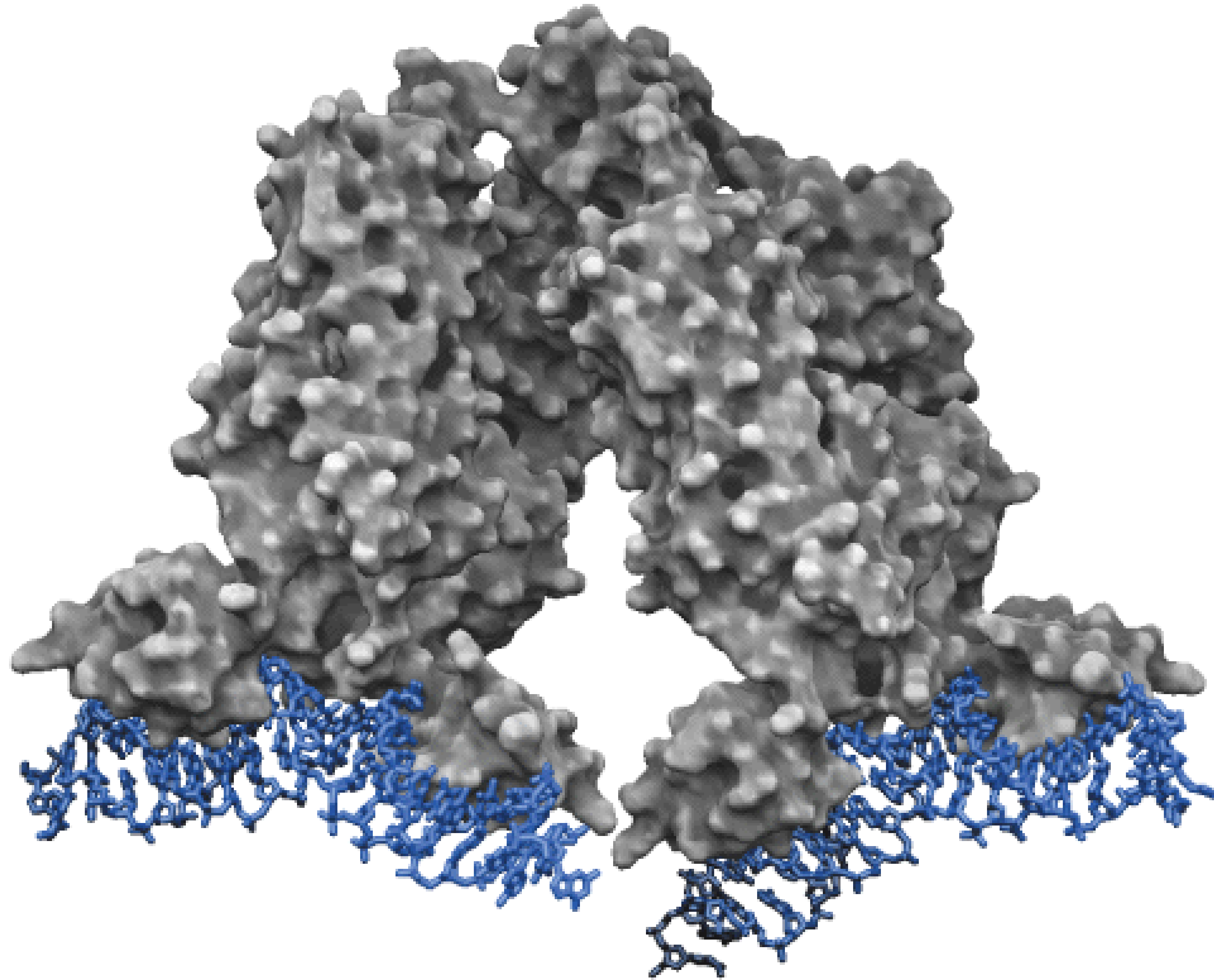
Lac repressor binds to the operators O_1, O_2 and O_3



Molecular structure of E. coli lac repressor dimer



Molecular structure of E. coli lac repressor tetramer



Lac repressor binding sites

	1				5					10		*					15					20	
O1	A	A	T	T	G	T	G	A	G	C		G		G	A	T	A	A	C	A	A	T	T
	T	T	A	A	C	A	C	T	C	G		C		C	T	A	T	T	G	T	T	A	A
O2	A	A	A	T	G	T	G	A	G	C		G		A	G	T	A	A	C	A	A	C	C
	T	T	T	A	C	A	C	T	C	G		C		T	C	A	T	T	G	T	T	G	G
O3	G	G	C	A	G	T	G	A	G	C		G		C	A	A	C	G	C	A	A	T	T
	C	C	G	T	C	A	C	T	C	G		C		G	T	T	G	C	G	T	T	A	A
SynL	A	A	T	T	G	T	G	A	G	C				G	C	T	C	A	C	A	A	T	T
	T	T	A	A	C	A	C	T	C	G				C	G	A	G	T	G	T	T	A	A
SynR	A	A	T	T	G	T	T	A	T	C	C		G	G	A	T	A	A	C	A	A	T	T
	T	T	A	A	C	A	A	T	A	G	G		C	C	T	A	T	T	G	T	T	A	A

Lac repressor head piece (1-62) bound to SynL sequence

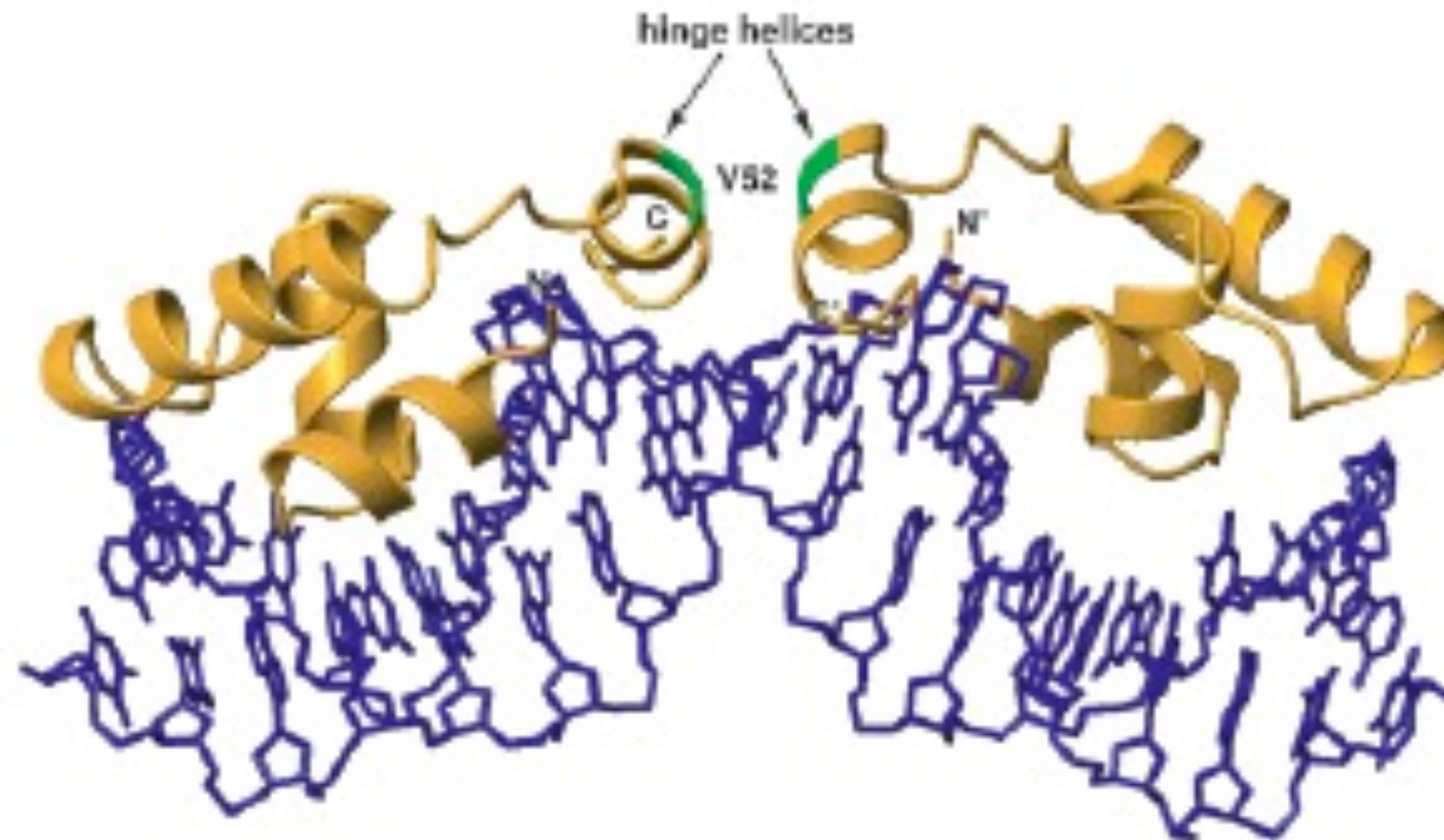


Figure 3. NMR solution structure of wild-type *lac* HP62 bound to the SymL operator (PDB access code 1CJG).[22] The two hinge helices (residues 50–58) bind to the minor groove of the SymL operator and bend significantly the DNA. On the basis of this structure, Val52 (green) was later replaced by a cysteine residue, so that a disulfide bond could link the two subunits, to yield a covalently linked dimeric lac that bound the natural operator *O1* with very high affinity.[35]

Lac repressor head piece (1-62) bound to the natural operator O1

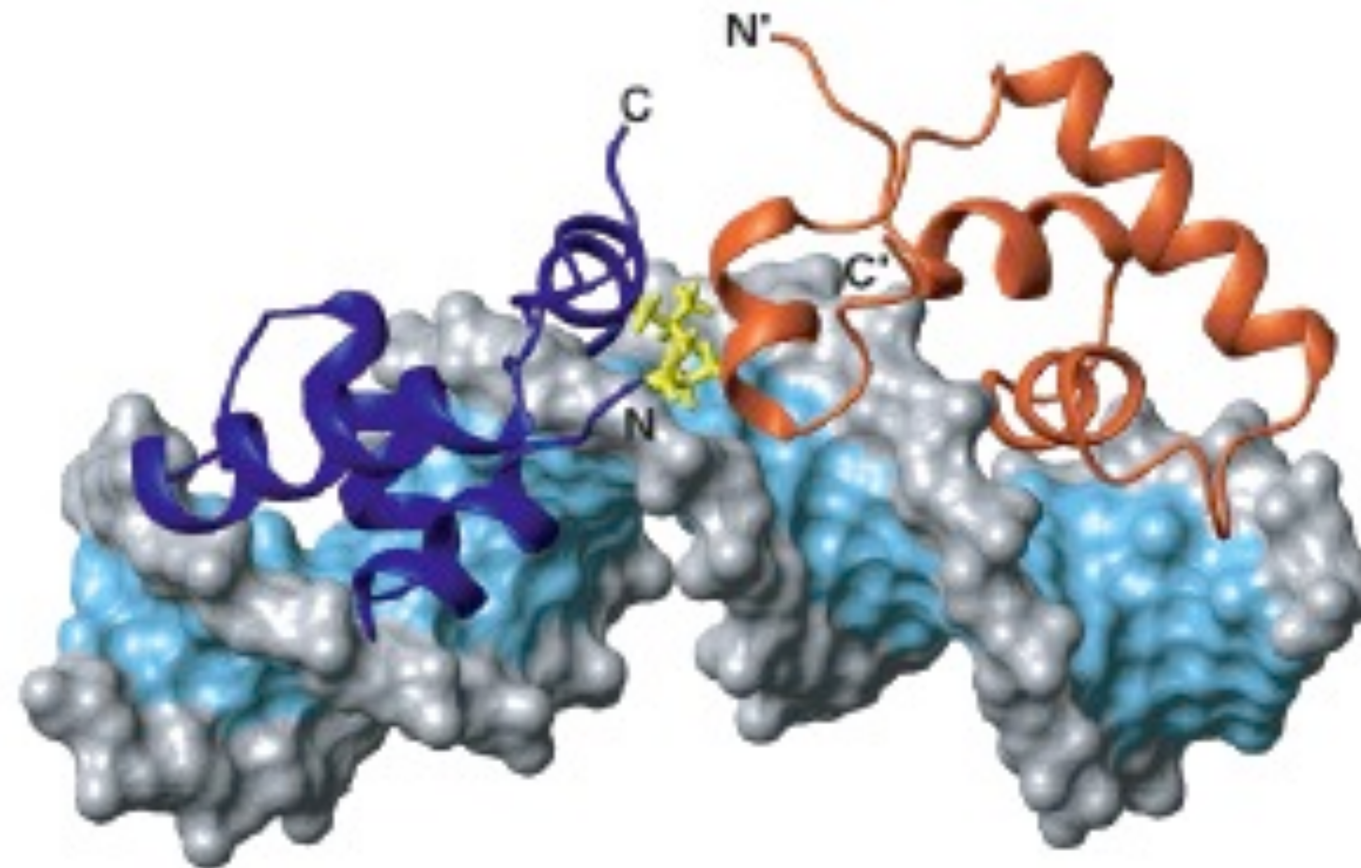
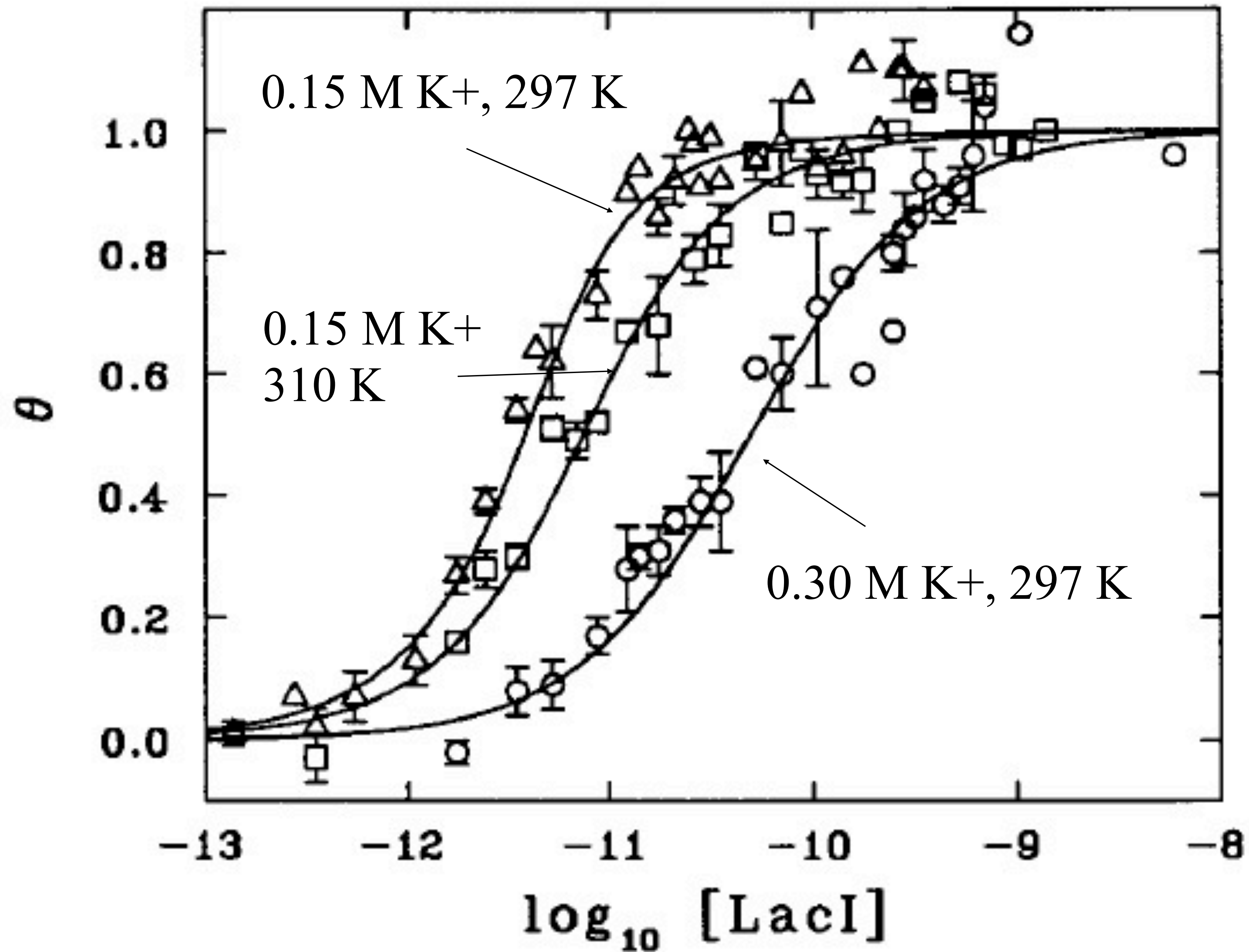
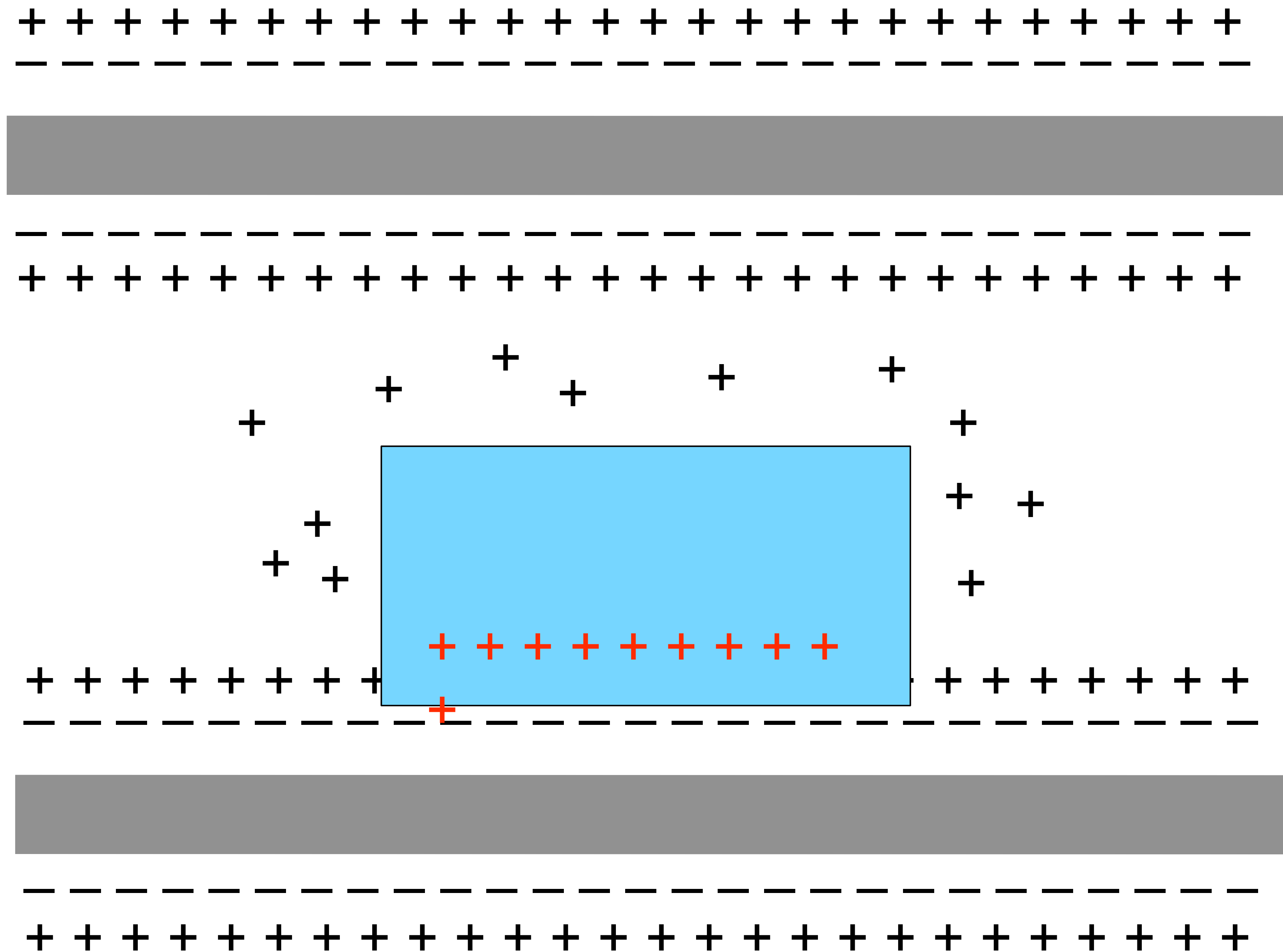


Figure 5. Three-dimensional structure of the dimeric *lac* DBD complexed to its natural *O1* operator (PDB accession code 1L1M).[41] A ribbon diagram of the protein is shown bound to the solvent-accessible surface of the operator. The left and right *lac* headpiece subunits are dark blue and dark orange, respectively. The major and minor grooves of the operator are light blue, and the ribose-phosphate backbone is gray. The side chains of Leu56 (shown in yellow) of both monomers protrude into the minor groove of the *O1* operator and introduce a ~36° kink centered between base pairs 10 and 11.

Binding titrations of symmetrical operator site with Lac repressor measured by filter binding assay



ΔS_{PE}: Favorable displacement of ions from the DNA



$$\Delta S_{PE} = 20 \text{ to } 60 \text{ cal K}^{-1} \text{ mol}^{-1}$$

The influence of binding one ligand (ion) on binding of another ligand (protein) to the DNA is referred to as binding "linkage"

Wyman Linkage Relationship

J. Wyman (1964) Adv. Protein Chem. 19, 223

$$\frac{\partial \log K}{\partial \log x} = \Delta\overline{X}$$

Electrostatic (Coulombic) Interactions

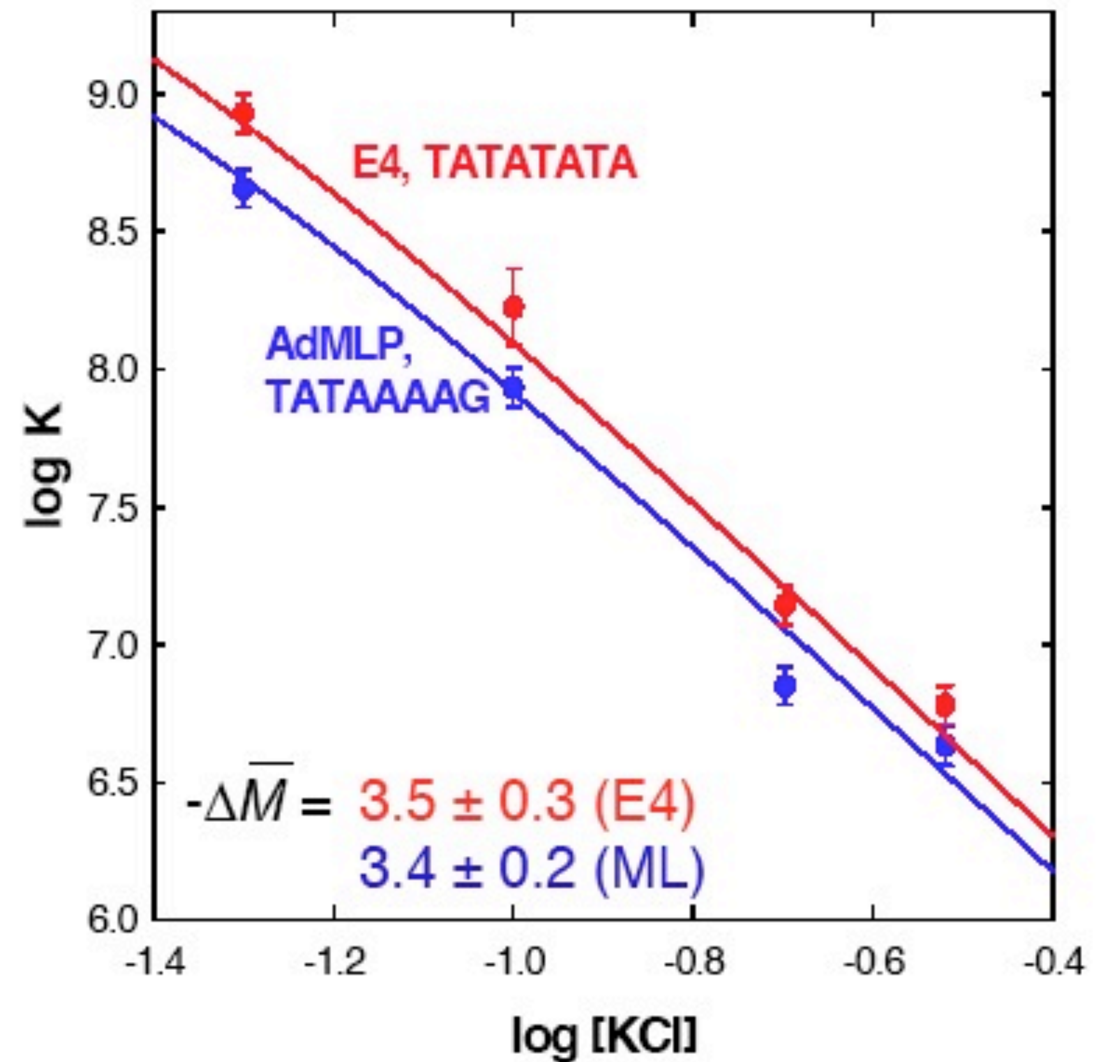
$$Interaction\ Energy = \frac{Z_a \cdot Z_b}{D \cdot r_{ab}}$$

Cation displacement

$$\frac{\partial \log K}{\partial \log[M^+]} = \Delta\overline{M}^+ = \varphi\overline{z}$$

fraction of charge neutralized = 0.88

number of salt bridges between DNA phosphates and positive amino acids



specific binding of TBP to different promoter sequences

Including the binding of ions in the reaction

Upon binding to the DNA *D* the protein *P* interacts with *n* phosphates so that $m = n \cdot \psi$ cations M^+ are displaced:

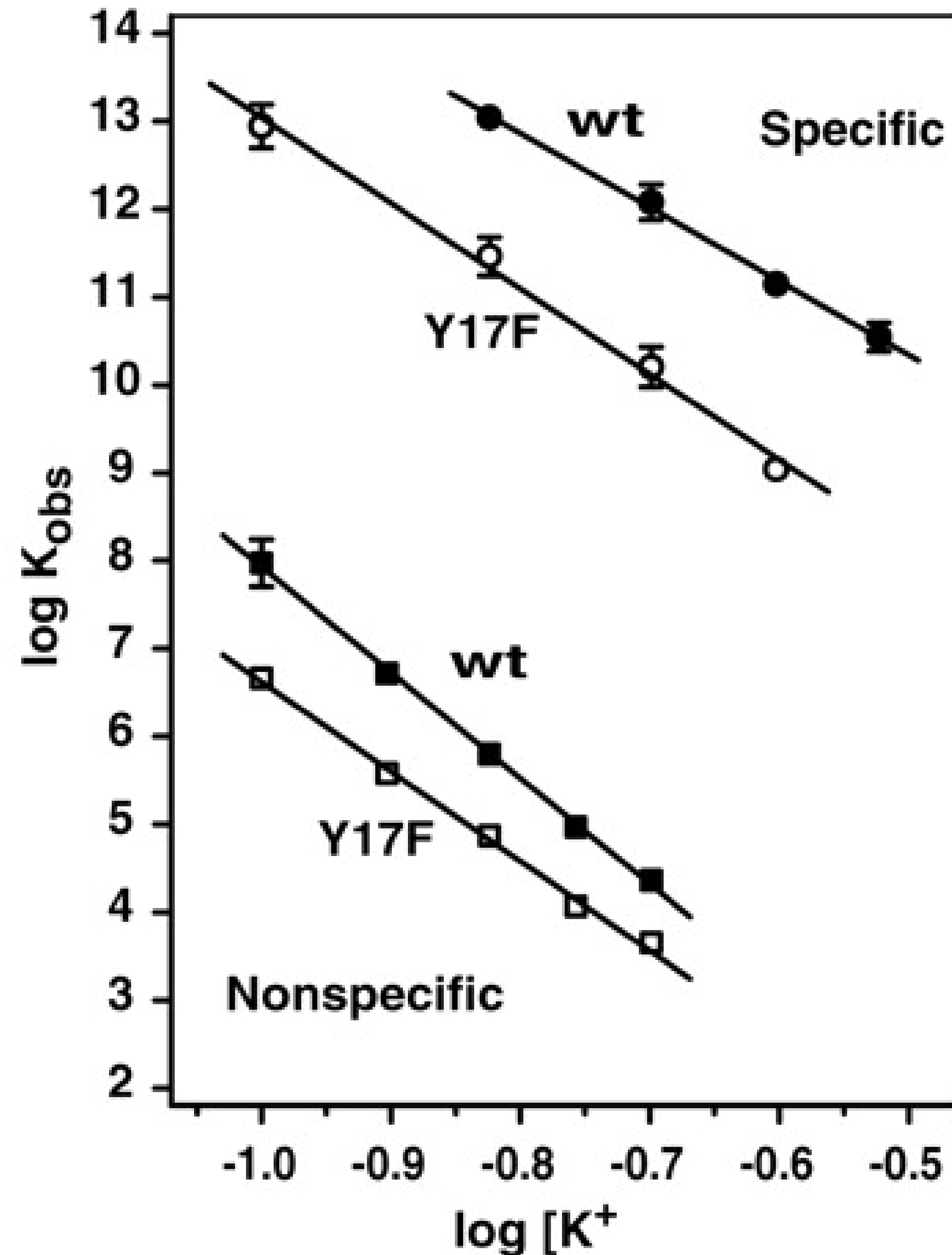
$$P + D \rightleftarrows PD + mM^+ \qquad K = \frac{PD \cdot \left[M^+\right]^m}{P \cdot D}$$

However, we derive K from the concentrations of P, D and PD, thus

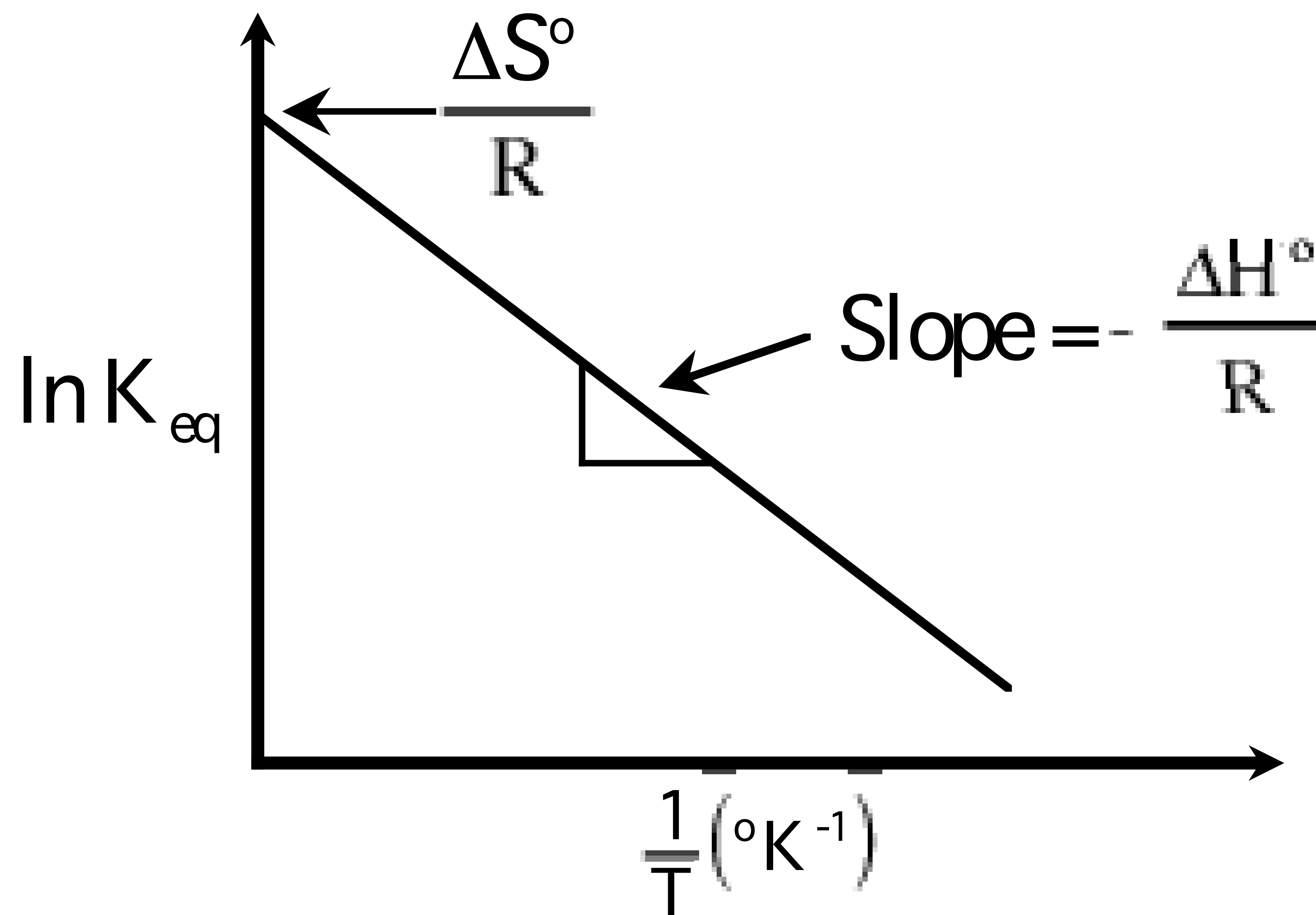
$$K_{obs} = \frac{\left[PD\right]}{\left[P\right] \cdot \left[D\right]} = \frac{K}{\left[M^+\right]^m}$$

$$\log K_{obs} = \log K - m \log \left[M^+\right]$$

Lac repressor binding involves 6-7 (specific) and 11 (nonspecific) charge-charge interactions



The temperature dependence of the binding constants reveals ΔH and ΔS in a van't Hoff plot if ΔH and ΔS are independent of temperature



$$\Delta G = \Delta \mathrm{H} - T \cdot \Delta S$$

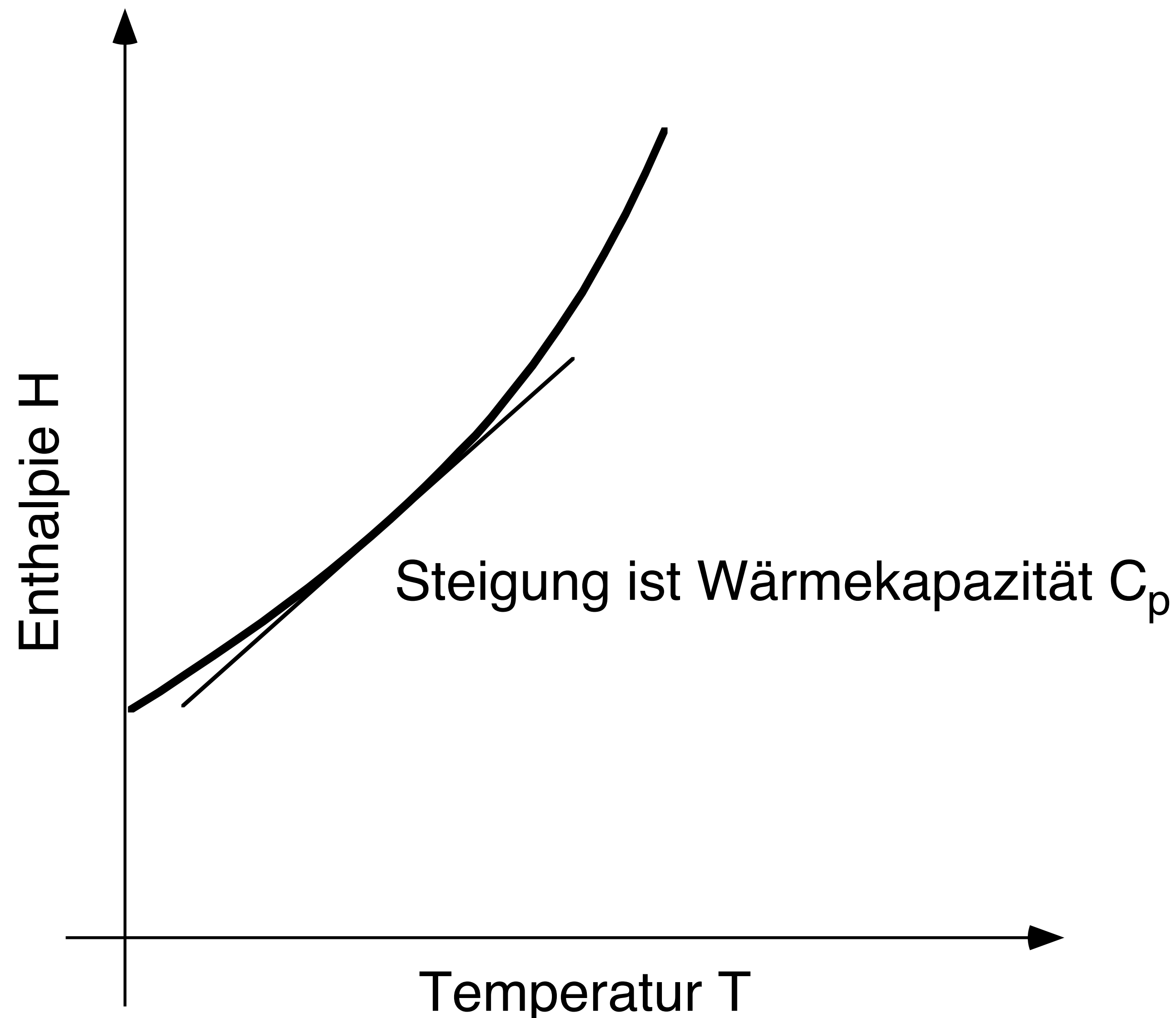
$$\Delta G = -R \cdot T \cdot \ln K_{\mathrm{eq}}$$

$$\ln K_{eq} = -\frac{\Delta H}{RT} + \frac{\Delta S}{R}$$

$$\frac{\partial(\ln K_{eq})}{\partial(1/T)} = -\frac{\Delta H}{R}$$

From the slope of ln K_{eq} vs. 1/T (usually from 0 to 40 °C) one can determine the ΔH and from extrapolation also ΔS . Is the van't Hoff plot curved then ΔH is temperature dependent and it can be determined from the derivative.

The heat capacity C_P describes the temperature dependence of ΔH and ΔS



$$C_P = \frac{\partial H}{\partial T} = \frac{T \partial S}{\partial T}$$

or if C_P is constant

$$H(T_2) - H(T_1) = C_P \cdot (T_2 - T_1)$$

which is a good approximation for the narrow interval from 0 to 40 °C usually studied

Relation between ΔC_P, ΔG and K_{eq} for binding

For two characteristic temperature T_H and T_S with

$$\Delta H(T_H) = 0 \text{ and } \Delta S(T_S) = 0 \quad \Rightarrow$$

$$\Delta H(T) = \Delta C_{\mathrm{P}} \cdot (T - T_H)$$

$$\Delta S(T) = \Delta C_{\mathrm{P}} \cdot \ln\left(\frac{T}{T_S}\right)$$

$$\Delta G(T) = \Delta C_{\mathrm{P}} \cdot (T - T_H) - T \cdot \Delta C_{\mathrm{P}} \cdot \ln\left(\frac{T}{T_S}\right)$$

$$\Leftrightarrow$$

$$\ln K_{eq} = \frac{\Delta C_{\mathrm{P}}}{R} \cdot \left[\frac{T_H}{T} - 1 - \ln\left(\frac{T_S}{T}\right)\right]$$

ΔC_p vs ΔA_{np} for protein folding

There is a linear correlation between the heat capacity change for protein unfolding and the buried non-polar surface area.

This relationship is identical to that seen for the transfer of hydrocarbons from aqueous solution to the pure liquid phase

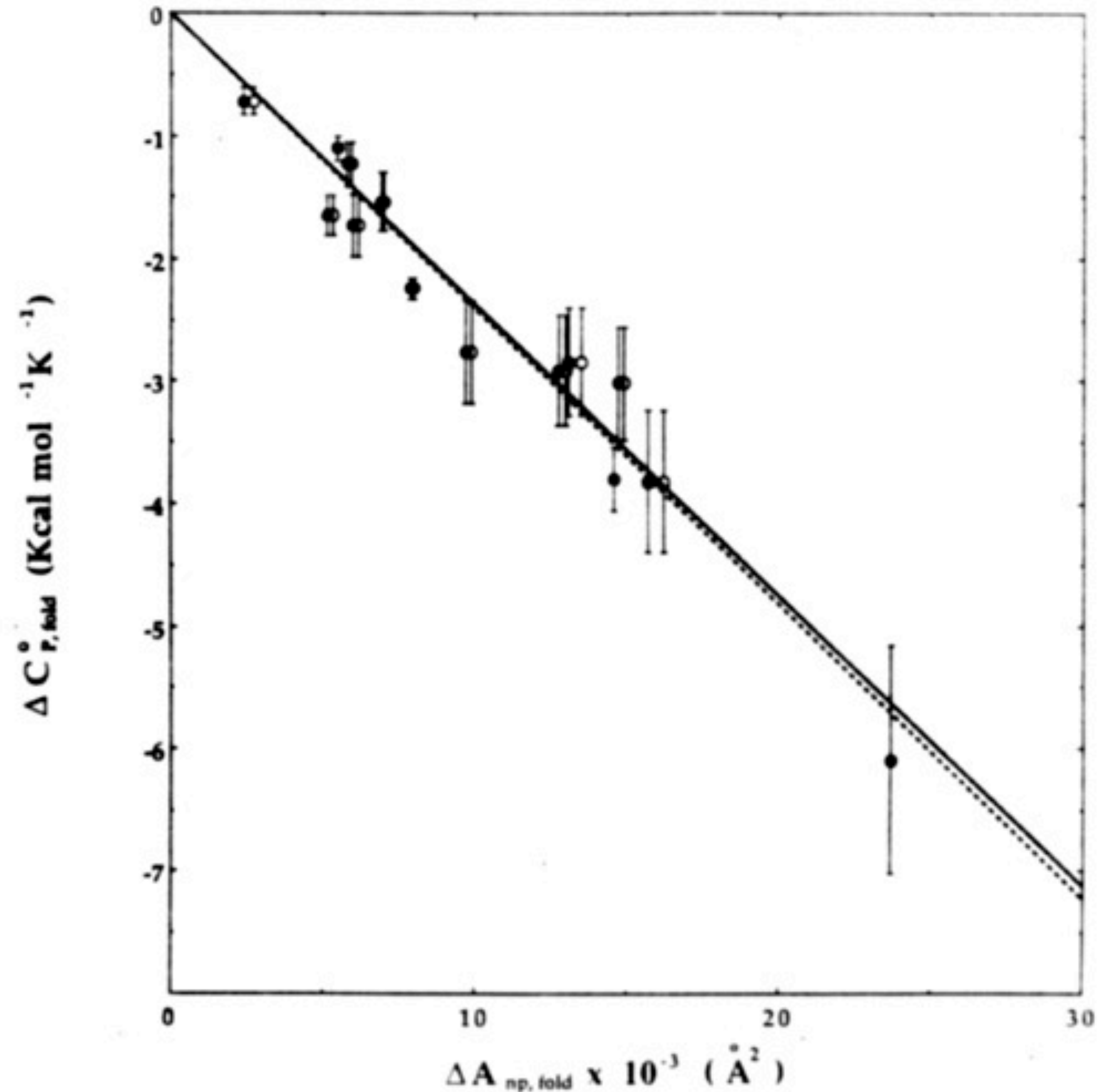
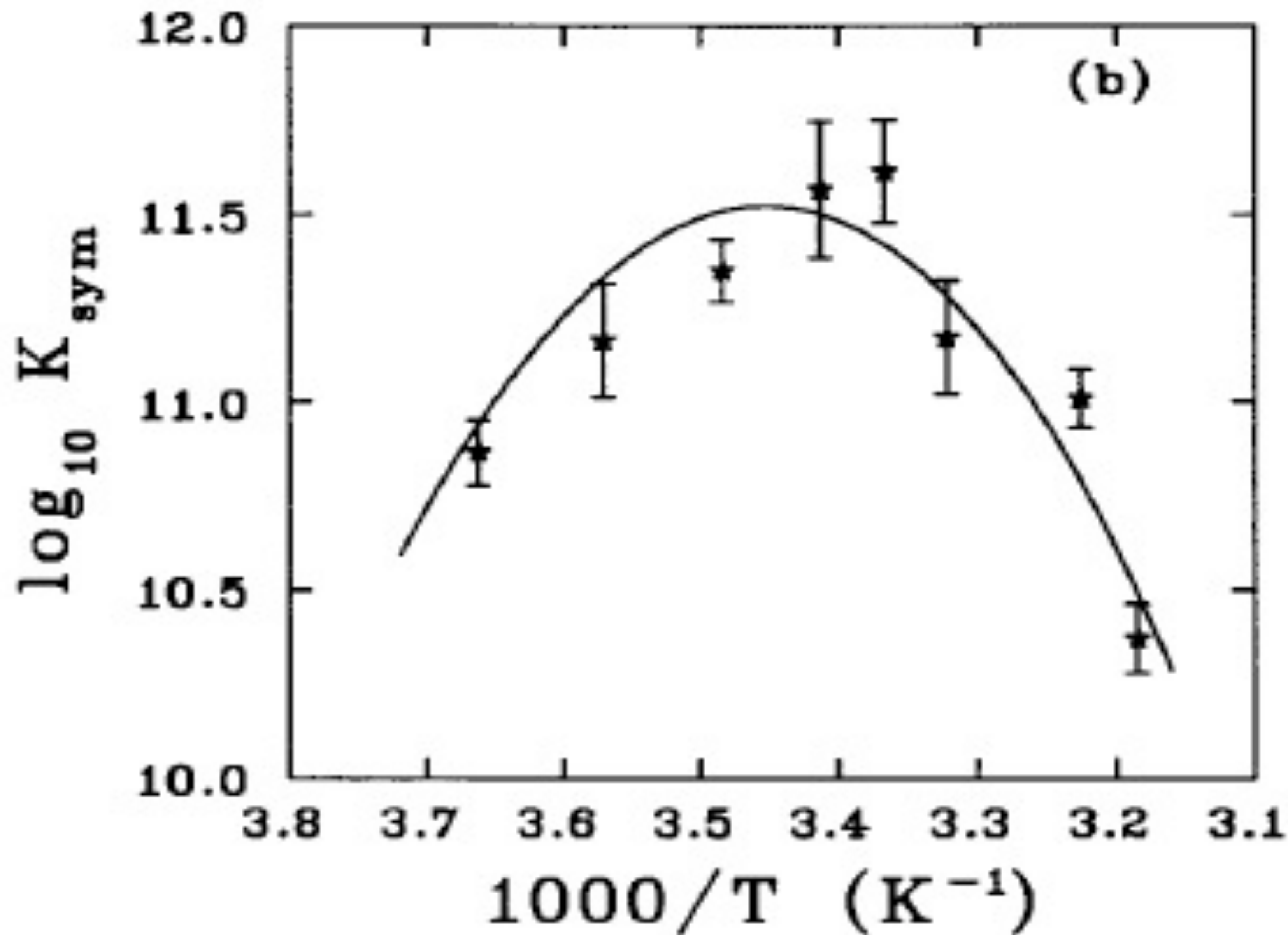


FIGURE 3: Standard heat capacity changes ($\Delta C^{\circ}_{p,fold}$) for the process of protein folding as a function of the reduction in water-accessible nonpolar surface area accompanying folding (ΔA_{np}). The denatured state is assumed to be in the extended β-form. The solid line is the weighted least-squares fit obtained by using set 1 radii (○) to calculate ΔA_{np}; the dashed line is the fit obtained by using set 2 radii (●). Where the two values of ΔA_{np} agree within the size of the data point, only one point (●) is plotted.

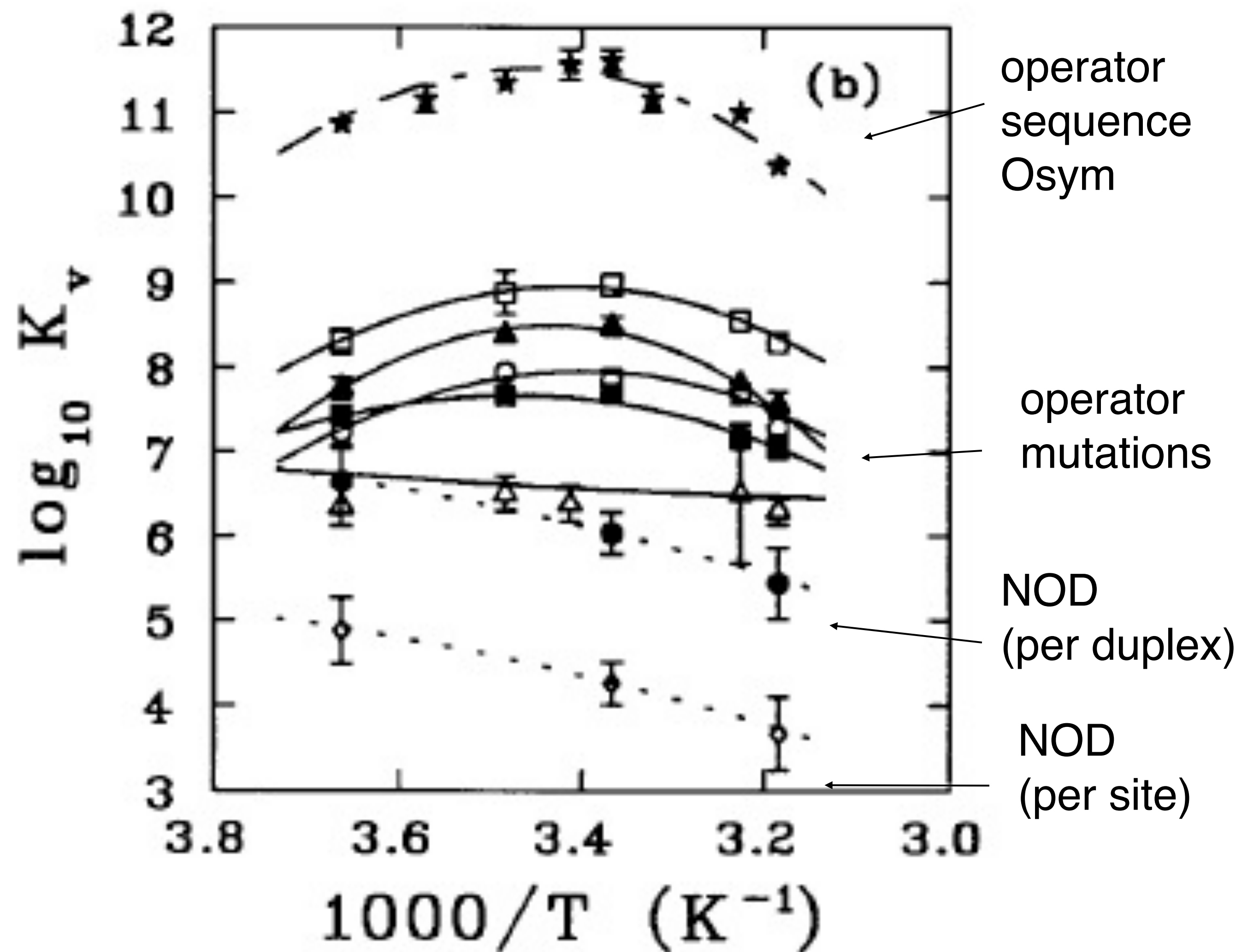
From Livingstone JR, Spolar RS, Record MT Jr. Biochemistry. 1991 Apr 30;30(17):4237-44

Temperature dependence of equilibirium binding constant for specific binding of lac repressor to the operator DNA



Temperature dependence of Kd for specific/nonspecific binding of lac repressor => less induced folding in the unspecific complex

specific binding vs.
unspecific binding



Osym Fragment:

10 9 8 7 6 5 4 3 2 1

5' GTAGTGGCGAAATTGTGAGCGCTCACAATTCGTTTGGCCG 3'

Variant Operators:

O4A AATTGTAAGCGCTTACAATT

O5A AATTGAGAGCGCTCTCAATT

O4A5A AATTGAAAGCGCTTTCAATT

O5C AATTGCGAGCGCTCGCAATT

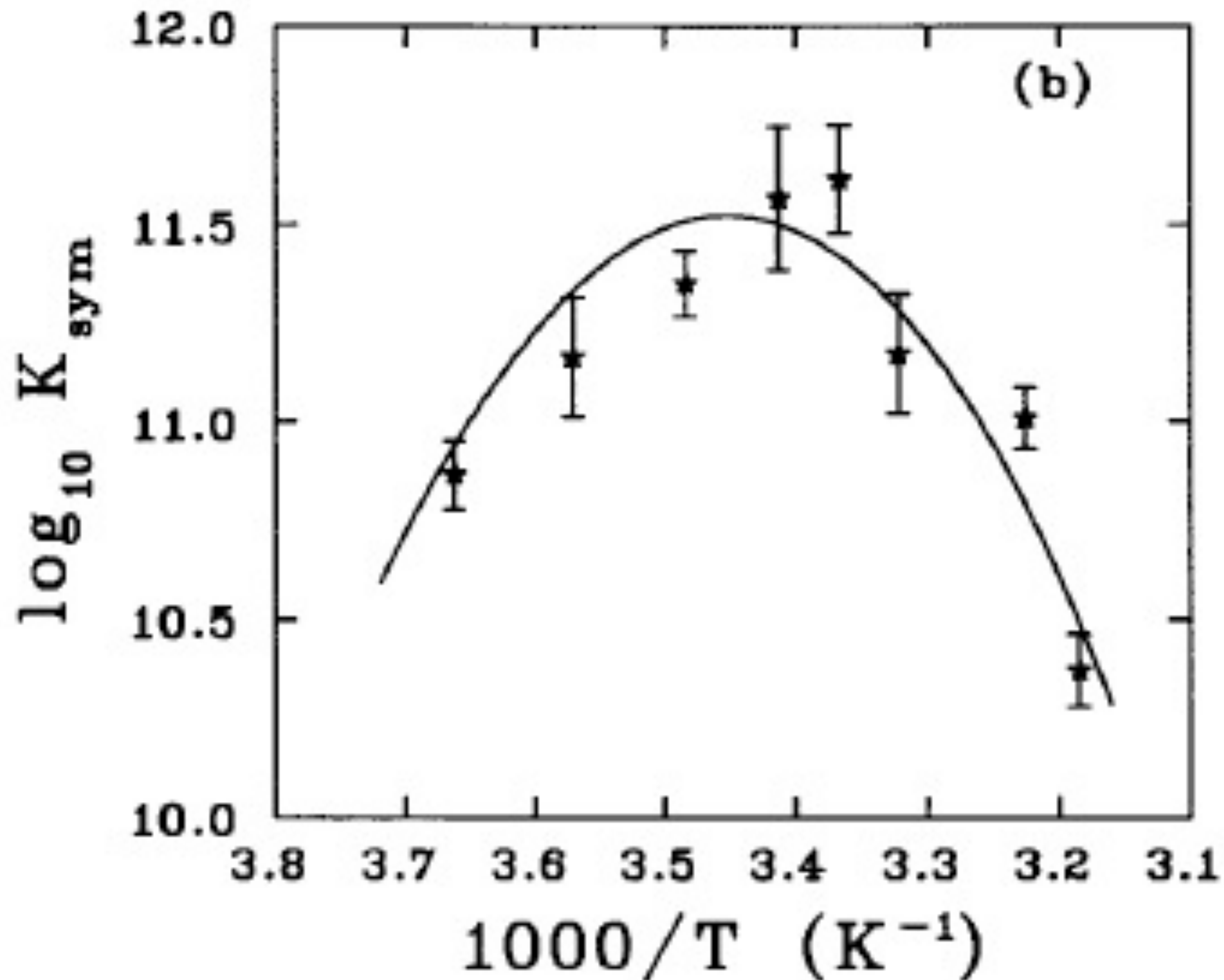
O4A5C AATTGCAAGCGCTTGCAATT

Nonoperator Fragment:

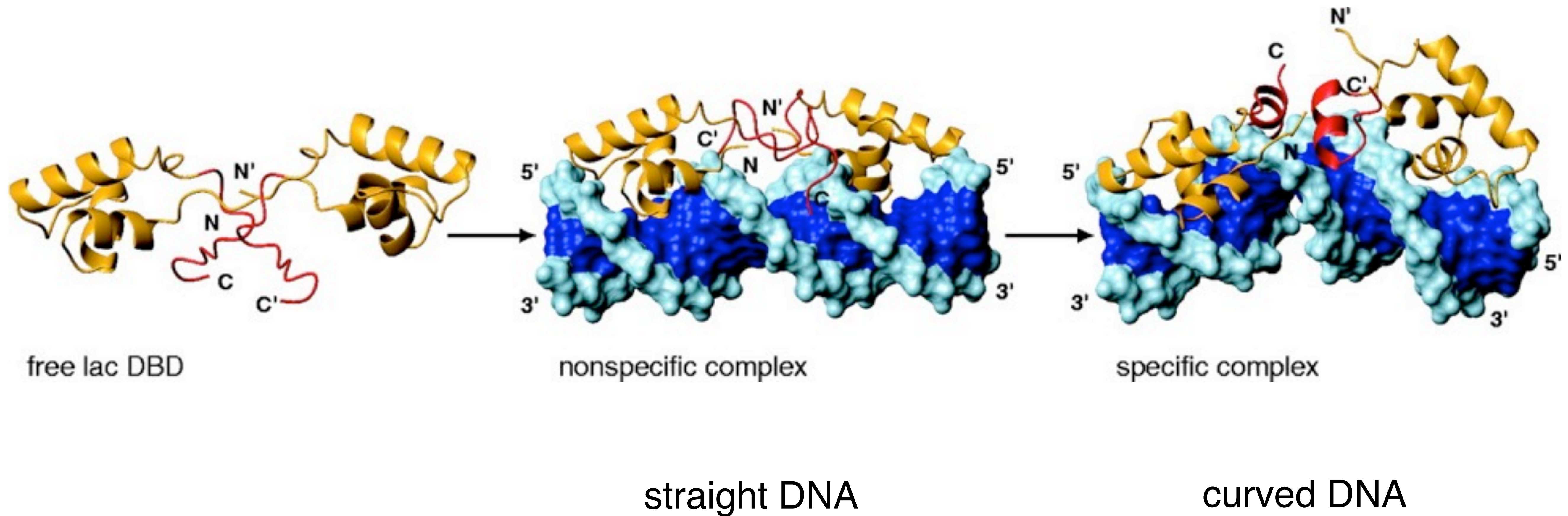
NOD TCTAAGAGTTACTCTATCCG

Temperature dependence of Kd for specific binding of LacI repressor => induced folding

specific binding to operator



The hinge region (50-62 in red) of Lac-DBD is folded only in the specific complex with DNA

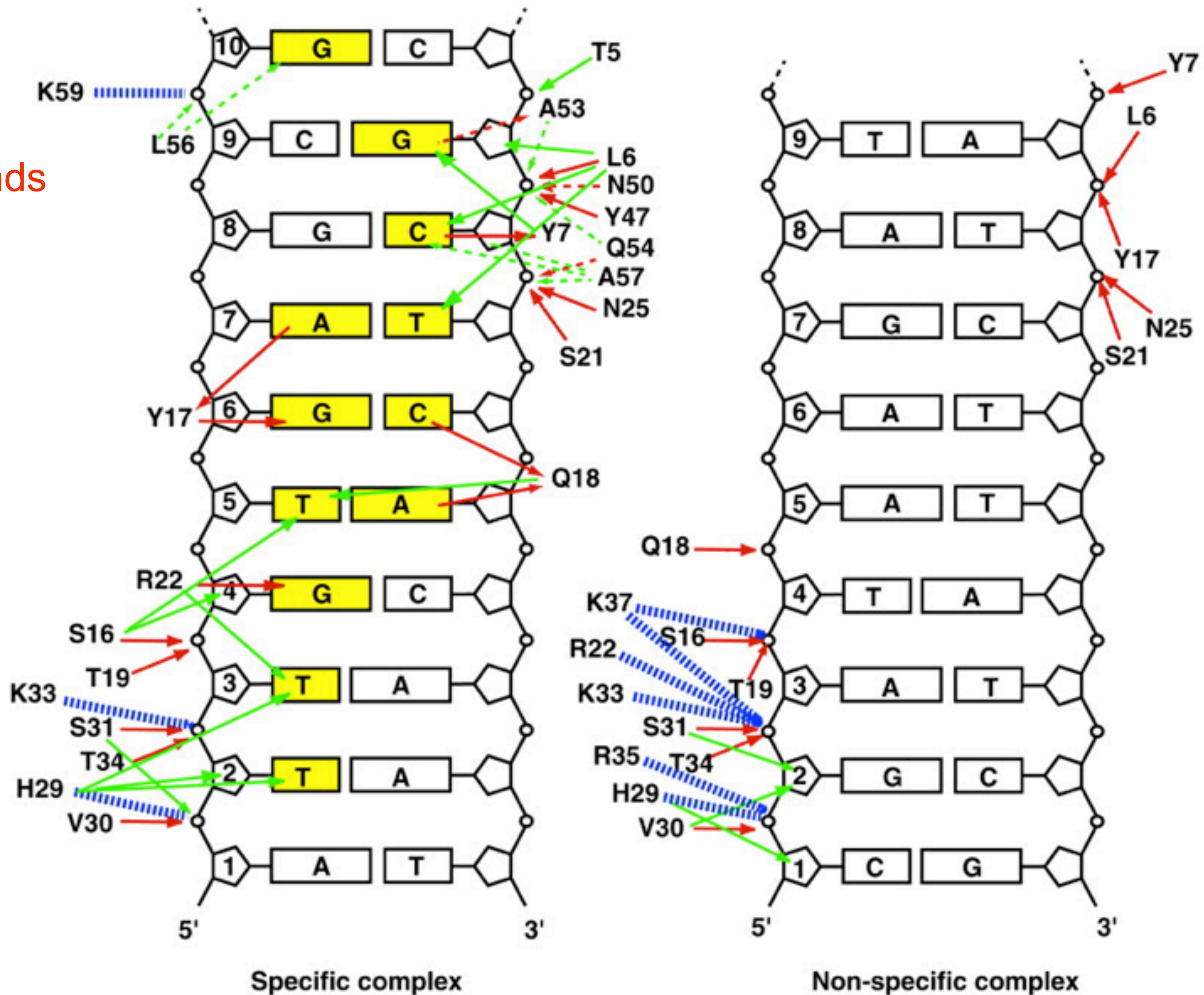


Specific (left) and nonspecific (right) protein-DNA contacts of Lac-DBD repressor with DNA

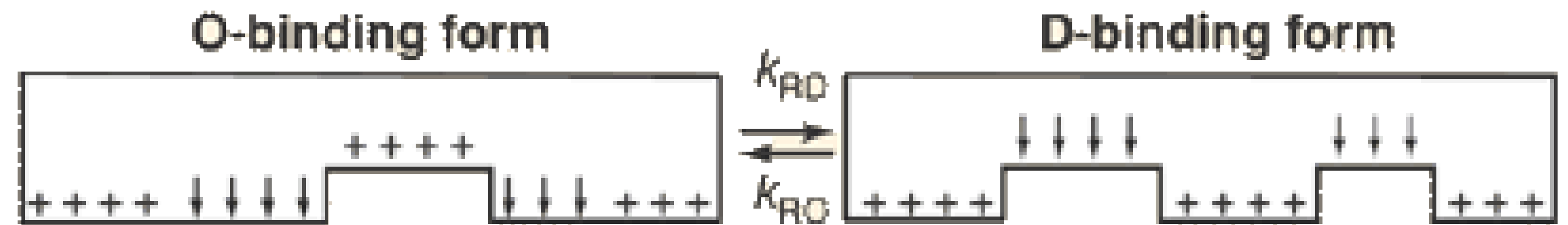
red:
hydrogen bonds

green:
hydrophobic contacts

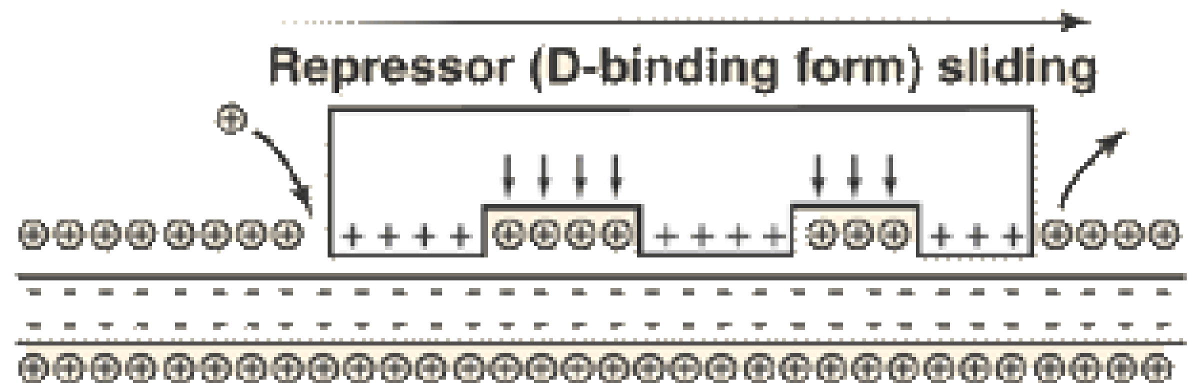
dashed blue:
electrostatic contacts



Schematic models of the specific (RO) and nonspecific (RD) complexes of Lac repressor



RO and RD conformations are dynamic and interconvert with rate constants k_{RO} and k_{RD} transient curvature of DNA also in unspecific complex?



- Small arrows denote specific hydrogen bonding in the protein binding site. That are established in the specific complex upon folding of the hinge region
- Plus signs (+) denote basic side chains located in and around the same site. In the “down” position these groups are in “interactive contact” with the underlying dsDNA, and in the “up” position these contacts are broken.
- RO complex: 7 hydrogen bonds with the base pairs of the DNA operator site, only 6 electrostatic interactions with the charged DNA backbones.
- RD complex: 11 charge-charge interactions with the dsDNA backbone, but all the specific interactions with the DNA base pairs have been “withdrawn.”
- curvature of DNA in the specific complex