doi:10.1016/j.jmb.2006.10.042 J. Mol. Biol. (2007) 365, 603–611

JMB

Available online at www.sciencedirect.com

ScienceDirect

ELSEVIER

Calorimetric Analysis of Binding of two Consecutive DNA Strands to RecA Protein Illuminates Mechanism for Recognition Of Homology

Masayuki Takahashi[1,2]*, Fabrice Maraboeuf[1], Katsumi Morimatsu[3] Tassadite Selmane[1], Fabrice Fleury[2] and Bengt Norden[3]

[1]*UMR 216, Centre National de la Recherche Scientifique and Institut Curie, F-91405 Orsay France*

[2]*UMR 6204, Centre National de la Recherche Scientifique and Université de Nantes, F-44322 Nantes cedex 3, France*

[3]*Department of Physical Chemistry, Chalmers University of Technology, S-41296 Gothenburg, Sweden*

**Corresponding author*

RecA protein recognises two complementary DNA strands for homologous recombination. To gain insight into the molecular mechanism, the thermodynamic parameters of the DNA binding have been characterised by isothermal calorimetry. Specifically, conformational changes of protein and DNA were searched for by measuring variations in enthalpy change (ΔH) with temperature (heat capacity change, ΔC_p). In the presence of the ATP analogue ATPγS, the ΔH for the binding of the first DNA strand depends upon temperature (large ΔC_p) and the type of buffer, in a way that is consistent with the organisation of disordered parts and the protonation of RecA upon complex formation. In contrast, the binding of the second DNA strand occurs without any pronounced ΔC_p, indicating the absence of further reorganisation of the RecA–DNA filament. In agreement with these findings, a significant change in the CD spectrum of RecA was observed only upon the binding of the first DNA strand. In the absence of nucleotide cofactor, the ΔH of DNA binding is almost independent of temperature, indicating a requirement for ATP in the reorganisation of RecA. When the second DNA strand is complementary to the first, the ΔH is larger than that for non-complementary DNA strand, but less than the ΔH of the annealing of the complementary DNA without RecA. This small ΔH could reflect a weak binding that may facilitate the dissociation of only partly complementary DNA and thus speed the search for complementary DNA. The ΔH of binding DNA sequences displaying strong base-base stacking is small for both the first and second binding DNA strand, suggesting that the second is also stretched upon interaction with RecA. These results support the proposal that the RecA protein restructures DNA, preparing it for the recognition of a complementary second DNA strand, and that the recognition is due mainly to direct base-base contacts between DNA strands.

Keywords: RecA protein; homologous recombination; isothermal titration calorimetry; DNA binding; thermodynamics

Introduction

The RecA protein plays a crucial role in homologous recombination in *Escherichia coli*:[1,2] it regulates the synthesis of proteins involved in the recombination reaction, including RecA itself, and catalyses the exchange of DNA strands. Purified RecA mimics these activities *in vitro* in the presence of cofactor ATP.[3–5] For these reactions, RecA first binds to a single-stranded DNA with high cooperativity and forms a nucleoprotein filament.[6,7] This

Present addresses: K. Morimatsu, University of California at Davis, Davis, CA 95616, USA; T. Selmane, Mount Sinai School of Medicine, New York, MY 10029, USA.

Abbreviations used: ATPγS, adenosine 5′-*O*-3-thiotriphosphate; ITC, isothermal titration calorimetry; poly(dεA)l, poly(1,N^6-etheno-deoxyadenosine).

E-mail address of the corresponding author: masayuki.takahashi@univ-nantes.fr

RecA-single-stranded DNA nucleofilament can then bind a second DNA molecule (either single or double-stranded), to pair complementary parts and catalyse strand exchange between them.[8,9] Eukaryotic homologues of RecA, such as Rad51, form a similar filament with DNA,[10–12] indicating that Rad51 uses a comparable reaction mechanism. RecA can thus be considered a prototypic recombinase and is for this reason studied extensively.

Kinetic analyses of the reaction,[9] and fluorescence measurements of probes attached to the DNA bases,[13] indicate that direct base–base interactions of the Watson–Crick type may occur between complementary DNA strands in the RecA filament and contribute to the recognition of homologous DNA. Structural analyses also indicate that RecA facilitates hydrogen bonding between the two DNA strands by stretching and stiffening the DNA,[6,14] restricting the local movements of DNA bases,[14,15] and orienting them in one direction.[14,16] The nucleobases of the second DNA strand are thus oriented almost coplanar with those of the first bound DNA strand, assisting their matching interaction.[16] The structure of the first DNA strand in the complex with RecA has been studied using NMR on a short oligonucleotide interacting with the RecA filament.[17] The nucleobases of the oligonucleotide are found to be unstacked and oriented, as concluded earlier for longer DNA from linear dichroism and fluorescence experiments.[6,14,16] On the basis of this DNA structure, a model for the strand exchange reaction has been proposed.[18] However, the structural details of the reaction intermediates are unknown.[19] Furthermore, the complex may undergo a large structural change during the reaction, and it is still unclear to what extent the direct contacts between the DNA strands are decisive for the recognition or "diagnostic contacts" of RecA with the DNA strands. To reach a better understanding of the mechanisms involved in the DNA recognition mediated by the RecA protein, we have determined the thermodynamic parameters of RecA–DNA interactions and, in particular, have considered the potential structural changes of RecA upon DNA binding, using isothermal titration calorimetry (ITC).

ITC measures directly the release or uptake of heat upon a reaction, i.e. the reaction enthalpy (ΔH) at a fixed temperature.[20,21] We have previously shown, by this approach, that the ΔH of binding of a second DNA strand to a pre-formed complex between RecA and a primary DNA strand depends upon the sequence complementarity with respect to the first DNA.[22] Here, we extend this type of study by performing experiments under systematically varied temperature and buffer conditions. Such analyses can provide useful structural information, including conformation changes, for model-building and drug design.[20,21] The variation of ΔH with temperature, which corresponds to a change in heat capacity (ΔC_p), can reveal a decrease in the accessible surface areas of the molecules upon complex formation.[23,24] This decrease is usually larger when disordered parts of the molecules become more structured upon interaction.[23,24] Structural changes of macromolecules can thus be detected. Since the flexible L1 and L2 loops of RecA are considered to be involved in DNA binding,[25–28] an increased degree of organisation of these parts upon DNA binding could be expected,[29] and thus associated with the appearance of a large ΔC_p.

In this study, we look for structural changes of RecA upon binding the first and second DNA strand by determining the variations in ΔH with temperature and we complement the investigation by performing CD measurements. In principle, the binding constant may be determined by ITC measurements, giving access to both the $\Delta G°$ and $\Delta S°$ of binding. However, an accurate determination of a binding constant for the RecA–DNA interaction is obviated by the fact that the oligomerisation of RecA in the absence of DNA must be taken into account.[7] Hence, we limit the analysis to determining the specific ΔH. Generally, the experiments were performed in the presence of adenosine 5′-*O*-3-thiotriphosphate (ATPγS) the non-hydrolysable analogue of ATP, because otherwise ATP hydrolysis during the experiment would severely perturb the calorimetric measurements. In addition, and conveniently, the reaction stops at the binding step of the second DNA strand in the presence of ATPγS without the release of the DNA strand.[30,31] We can thus measure directly the binding enthalpy by experiments performed in the presence of ATPγS. Moreover, in the presence of ATPγS, a non-complementary DNA strand can be bound to the second site of RecA but with less affinity than the complementary DNA strand.[30] Some experiments were performed in the absence of nucleotide to understand the role of the nucleotide cofactor in the reaction.

Results

Temperature-dependence of ΔH for complex formation of RecA with DNA

We performed calorimetric titrations at different temperatures in order to assess any changes in heat capacity, ΔC_p, between reaction and product species involved in the formation of the RecA–DNA complex. To avoid the formation of aggregates, we carried out the experiments with short oligonucleotides of 32 bases (or base-pairs).[16] Typical isothermal titrations of RecA with oligo(dT) in the presence and in the absence of ATPγS are shown in Figure 1. In the presence of ATPγS, when RecA can bind two DNA molecules with a stoichiometry of three bases per RecA subunit for each DNA strand,[14] we observed two steps of ΔH: the ΔH per added amount of DNA was large and nearly constant up to a ratio of three DNA bases/RecA monomer, while above this ratio it became smaller but was almost constant in the interval between three and six DNA bases/RecA monomer (Figure 1(a)). Beyond six DNA bases/

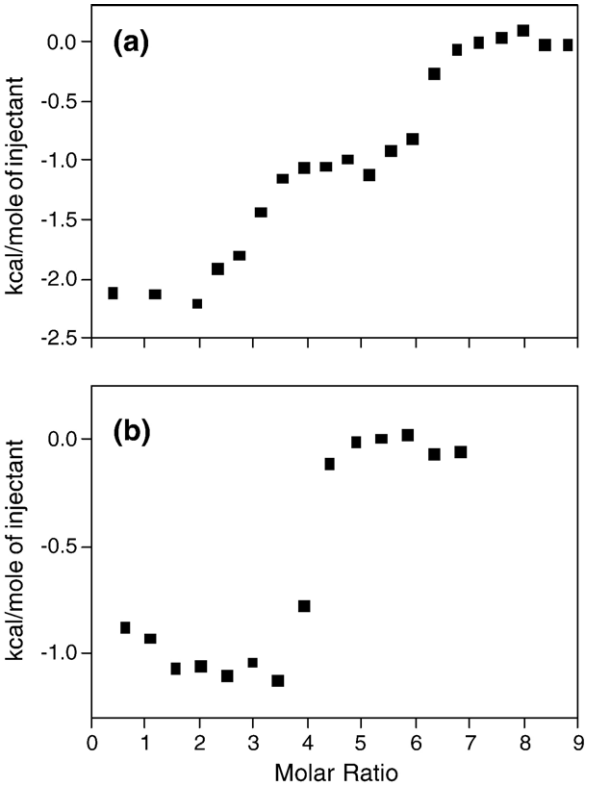

Figure 1. Example of isothermal titration of RecA by oligo(dT). Isothermal calorimetry (ITC) measurements were performed at 25 °C and pH 7.4 by injecting oligo (dT) in steps to 2 μM RecA (a) in the presence and (b) in the absence of 80 μM ATPγS.

RecA, the heat dissipation was very small or insignificant. The first, larger heat release of reaction corresponds to the binding of the first DNA, while the second, smaller heat release is due to the binding of the second DNA. In the absence of nucleotide cofactor, when RecA binds only one DNA molecule,[14] we observed only a single ΔH step: the ΔH per added amount of DNA was large and almost constant up to a ratio of four DNA bases/RecA monomer, while the reaction heat was insignificant above this ratio (Figure 1(b)).

The ΔH of the binding of DNA in absence of the nucleotide cofactor was almost independent of temperature (Figure 2). ΔC_p was then only −20 cal/mol of RecA monomer K^{-1}, a typical value for a non-specific DNA–protein interaction,[32] indicating an absence of reorganisation of RecA, as well as DNA, in the complex formation. In contrast, the ΔH of the binding of the first DNA strand in the presence of ATPγS depended strongly upon the temperature and increased in amplitude approximately linearly with temperature (Figure 2), giving a ΔC_p of −600 cal mol^{-1} K^{-1}. This result suggests some reorganisation of RecA and/or DNA. The ΔH for the binding of the second DNA strand showed considerably less dependence upon the temperature and ΔC_p here was only −100 cal mol^{-1} K^{-1} (Figure 2). There may be much less structural change upon binding the second DNA strand. Supporting this conclusion is the observation that the CD spectrum of RecA was affected significantly by the addition of oligo(dT) but only up to a stoichiometry of three bases per RecA subunit with ATPγS (corresponding to the saturation of the first DNA-binding site),[33] while no significant change in CD was observed upon adding oligo(dT) in excess of this stoichiometry (corresponding to the binding of the second DNA molecule) or binding without nucleotide cofactor (data not shown).

Very similar results (regarding ΔH and ΔC_p for binding both the first and second DNA strands) were obtained when poly(dT), some 200 bases long, was used (Table 1), indicating that the conclusion obtained with short oligonucleotides is valid also for the binding of longer DNA. Since the binding of RecA to DNA depends on DNA sequence (or DNA conformation), we performed the same experiments using two other oligonucleotides, oligo(dA) and oligo(dG-dT). The binding of oligo(dA), which is the least favoured,[22,34,35] most likely because of its pronounced base self-stacking as a single strand,[36] displayed smaller enthalpies for the binding of both the first and the second DNA strand as compared to the corresponding enthalpies of oligo(dT) binding (Table 1). This observation suggests that unstacking of nucleobases occurs upon the binding of both the first and the second DNA strand. The binding of oligo(dG-dT), which is a favoured DNA sequence for RecA interaction,[34,35] exhibited a ΔH similar in amplitude to that of oligo(dT) (Table 1). Somewhat surprisingly, the temperature-dependence was almost independent of DNA sequence (Table 1): about −600 cal mol^{-1} K^{-1} for the first DNA strand and about −100 cal mol^{-1} K^{-1} for the second DNA

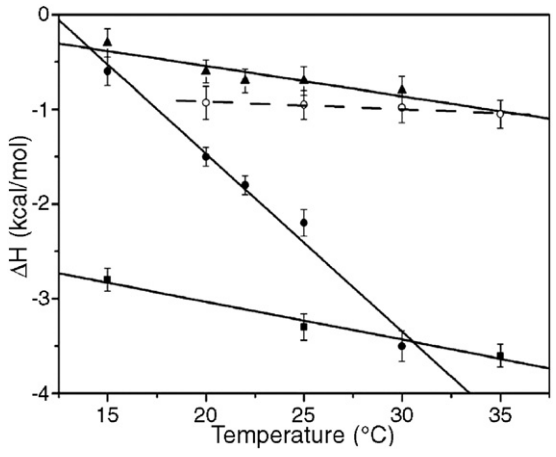

Figure 2. Change in binding enthalpy of first and second oligo(dT) to RecA with temperature. ΔH of binding of first oligo(dT) to free RecA in the presence (filled circles) and in the absence (open circles) of ATPγS, and that of second oligo(dT) to the RecA-oligo(dT) complex (triangles) and the RecA-oligo(dA) complex (squares) in the presence of ATPγS were determined from ITC measurements, as shown in Figure 1, at various temperatures. They are presented as a function of temperature to estimate the ΔC_p of each reaction.

Table 1. Effect of DNA sequence on the enthalpy (ΔH) and heat capacity (ΔC_p) changes for the binding of the first and second DNA to RecA at 25 °C

DNA added	Solution	ΔH (kcal/mol)	ΔC_p (cal $mol^{-1}K^{-1}$)
A. *First DNA binding*			
Oligo(dT)	RecA	−2.2±0.14	−600±42
Oligo(dA)	RecA	−0.5±0.17	−660±53
Oligo(dG-dT)	RecA	−1.9±0.13	−580±48
Poly(dT)	RecA	−2.4±0.15	−600±45
B. *Second DNA binding*			
Oligo(dT)	RecA/(dT)	−0.9±0.15	−100±24
Oligo(dA)	RecA/(dA)	−0.2±0.16	n.d.
Oligo(dG-dT)	RecA/oligo(dG-dT)	−0.4±0.14	−110±22
Oligo(dT)	RecA/(dA)	−3.3±0.13	−120±10
Oligo(dA)	RecA/(dT)	−1.9±0.15	−160±18
Poly(dT)	RecA/(dT)	−0.6±0.15	−90±24
Oligo(dA):(dT)	RecA/(dA)	−0.4±0.16	n.d.
Oligo(dA):(dT)	RecA/(dT)	−1.0±0.13	−40±32
Oligo(dA):(dT)	RecA/oligo(dG-dT)	no binding	n.d.
Oligo(dG-dT):(dC-dA)	RecA/oligo(dG-dT)	−0.5±0.17	−30±28
C. *Without ATPγS*			
Oligo(dT)	RecA	−1.1±0.16	−20±19
D. *Without RecA*			
Oligo(dT)	oligo(dA)	−7.0±0.13	n.d.

n.d.: not determined.

strand in all cases. The ΔC_p for the binding of the first oligo(dA), which is expected to undergo a larger structural change when passing from the pronounced stacked free state to the presumably unstacked bound state, was thus similar to that of oligo(dT) (−660 cal mol^{-1} K^{-1} *versus* −600 cal mol^{-1} K^{-1}). This observation suggests that the large ΔC_p of binding of the first DNA strand is mainly due to conformational changes and interactions of the protein, although some contribution from conformational changes of DNA cannot be excluded.

ΔH for binding of complementary second DNA and its temperature-dependence

We have observed that the enthalpy of binding of a second DNA strand depends upon the sequence of the first DNA and is larger when the DNAs are complementary.[22] We measured, at systematically varied temperature, the ΔH of binding oligo(dT) to a pre-formed RecA–oligo(dA) complex and compared it with that of oligo(dT) to a pre-formed RecA–oligo (dT) complex. We performed the same analysis for the binding of oligo(dA). As observed previously, the enthalpies for binding a second DNA strand were larger in amplitude when the first DNA strand was complementary to the second one (Table 1). However, in all cases, the temperature-dependence of ΔH (i.e. ΔC_p) was small and similar to that of the binding of a non-complementary second single-stranded DNA (Table 1).

The study showed also that the ΔH of binding of oligo(dT) to the complementary RecA–oligo(dA) complex is greater in amplitude than that of oligo (dA) binding to the complementary RecA–oligo (dT) complex (Table 1). The binding of oligo(dA) to the second site was also less favoured than that of oligo(dT) in comparable situations. At the same time, the ΔC_p for the binding of oligo(dA) as the second DNA strand was larger than that for poly (dT) (−160 *versus* −120 cal/mol/°K), like in the case of binding oligo(dA) as the first DNA strand (−660 *versus* −600 cal mol^{-1} K^{-1}) (Table 1). This difference may be related to some additional structural changes for binding oligo(dA), such as the unstacking of DNA bases.[37]

For comparison, we measured the ΔH of annealing of oligo(dT)/oligo(dA) in the absence of RecA by adding oligo(dT) to an oligo(dA) solution. We obtained a ΔH of −7 kcal mol^{-1} of bases (Table 1). This value is much larger than that for the binding of oligo(dA) to the RecA–oligo(dT) complex. The hydrogen bonds between the bases of oligo(dT) and oligo(dA) may thus be regarded as considerably weakened by RecA. This conclusion is in agreement with the notion that the hydrogen bonds of a nucleic acid double helix derive their strength from the hydrophobic environment of stacked bases.

Finally, the binding of double-stranded DNA to the second site of RecA was examined. The binding of oligo(dA):(dT) to the RecA–oligo(dT) or RecA–oligo(dA) complex was exothermic, like the binding of single-stranded complementary DNA, but presented a smaller ΔH (Table 1). We did not observe any significant ΔH signal upon adding oligo(dA):(dT) to the non-complementary RecA–oligo(dG-dT) complex, reflecting an absence of interaction. This was confirmed by linear dichroism measurement: the addition of oligo(dA):(dT) double-stranded DNA to the RecA–oligo(dT-dG) complex did not affect the linear dichroism signal, while that to the RecA–oligo(dT) or RecA–oligo (dA) complex showed an additional negative linear dichroism signal around 260 nm (data not shown). In contrast, when oligo(dT-dG):(dA-dC) double-stranded DNA was added to the complementary RecA–oligo(dT-dG) complex, a ΔH of −0.5 kcal mol^{-1} was observed, supporting the notion that a marked enthalpy is associated with the recognition of complementarity. The ΔH varied only slightly with temperature (Table 1), indicating an absence of a significant reorganisation of the RecA-DNA filament for the binding of the second DNA, also in the case of complementary double-stranded DNA.

Effect of pH

Binding of RecA to DNA is favoured by a pH below neutral.[22,38,39] Fluorescence measurements show that the binding of RecA to fluorescein-labelled oligo(dA) is faster at pH 6.2 than at pH 7.4 (data not shown), as was also observed by Chabbert and colleagues for RecA binding to poly(dεA).[38] This pH-dependence could be the result of some pH-dependent structural change of RecA: the protein might be more structured at the lower pH, and this

structure may be similar to that in the final complex, and thus less structural change would be required for DNA binding. In this case, a smaller ΔC_p at lower pH would be expected because of the smaller structural rearrangement of RecA for DNA binding. To investigate this hypothesis, we performed calorimetric measurements at pH 6.2 to compare them with those observed at pH 7.4.

Interestingly, the temperature-dependence of ΔH for the binding of the first DNA was significantly less at lower pH (Figure 3). ΔC_p was -420 cal mol^{-1} K^{-1} at pH 6.2 but as much as -600 cal mol^{-1} K^{-1} at pH 7.4. This suggests that less organisation of RecA upon DNA binding is required at lower pH so there could be some pH-dependent conformational change of RecA. Careful inspection of the CD spectrum of RecA showed that a negative band around 210 nm was somewhat larger at pH 6.2 than at pH 7.4 (Figure 4), supporting this proposition. The observation that pH affects both ΔC_p and the structure of RecA (in the absence of DNA) further supports the conclusion that the large ΔC_p detected for the binding of the first DNA is mainly due to conformational changes of RecA, including the effects of RecA–RecA interactions. The ΔC_p for the binding of the second DNA was, within experimental error, independent of pH (Figure 3).

Effect of buffer

We examined the possibility of proton uptake (or release) upon complex formation. Calorimetry measures the entire enthalpy change of a reaction, including solvent and ion-exchange, protonation of buffer by protons released upon complex formation (or deprotonation of the buffer by uptake of protons by the complex), etc.[40] Since the enthalpy of protonation of a buffer depends upon its nature, one can obtain an estimate of the number of protons released or involved upon complex formation by performing the reaction in various buffers. We carried out ITC measurements of RecA–oligo(dT) interactions in Tris–HCl and in cacodylate buffer, as well as in the phosphate buffer. The enthalpy of binding the first DNA strand was found to be much smaller in amplitude in Tris–HCl buffer than in

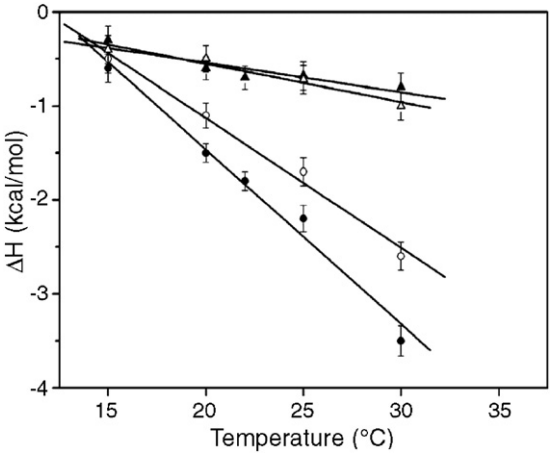

Figure 3. Effect of pH on enthalpy and heat capacity change of the binding of oligo(dT) to RecA. ΔH of binding of first (circles) and second (triangles) oligo(dT) to RecA with ATPγS were determined at various temperatures at pH 6.2 (open symbols) and pH 7.4 (filled symbols), as noted in Figure 2. They are presented as a function of temperature.

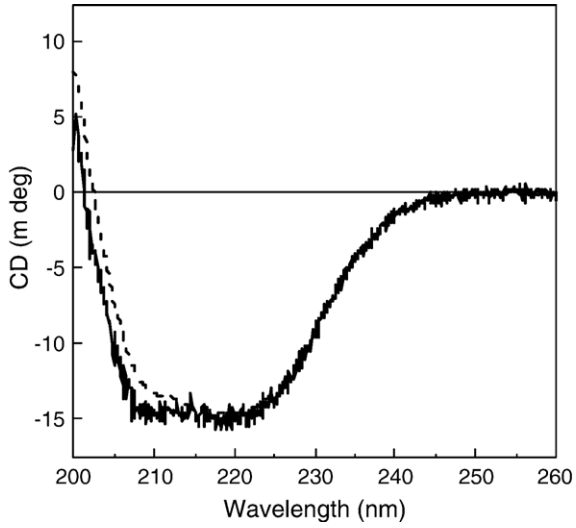

Figure 4. Effect of pH on the secondary structure of RecA. CD spectra of 1 μM RecA were measured at 25 °C at pH 6.4 (continuous line) and at pH 7.4 (broken line). The pathlength of the cell was 0.2 cm.

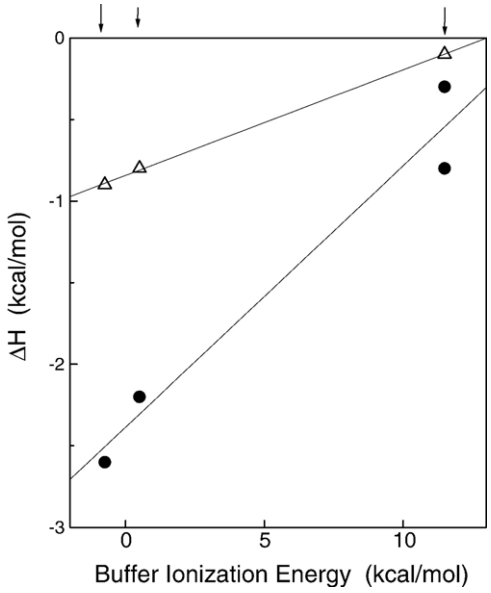

Figure 5. Proton uptake upon binding of oligo(dT) to RecA. To estimate the proton uptake of the reaction, ΔH of binding of first (filled circles) and second (open triangles) oligo(dT) to RecA were determined by ITC measurements at pH 7.4 in Tris–HCl, phosphate, and cacodylate buffers. They are presented as a function of the ionisation enthalpy of the buffer (Tris: 11.3 kcal mol^{-1}; phosphate: 1.2 kcal mol^{-1}; cacodylate: -0.55 kcal mol^{-1}).

phosphate buffer at pH 7.4 (Figure 5). This buffer dependence is not due to a difference in the binding mode of the buffer, because a salt back-titration experiment of the ATP–RecA–poly(dεA) complex showed no significant difference according to the nature of the buffer (data not shown). From these data, it can be concluded that there is an uptake of about 0.5 proton per RecA monomer upon binding of the first DNA strand.

Only a minor dependence on the choice of buffer was observed for the binding of the second DNA strand (Figure 5), indicating an absence of further protonation of RecA upon the binding of the second DNA strand. This is the case also for the binding of a complementary, second DNA strand. The binding of oligo(dT) to a pre-formed complementary RecA–oligo(dA) complex appears almost independent of the nature of the buffer (data not shown). This indicates that the recognition of complementary DNA may not involve any net uptake of protons.

Discussion

The present calorimetric characterisation of RecA–DNA interactions shows that the recognition of a complementary strand of DNA is favoured by enthalpy, but not associated with any large heat capacity change, just like the binding of non-complementary DNA. Thus, the recognition of a complementary DNA does not seem to require any additional structural change of the RecA filament. Our results support the proposal that RecA plays the role of a scaffold for the DNA strands, and that the recognition occurs mainly through hydrogen bonding between complementary DNA bases. Our previous fluorescence analysis indicates that the DNA bases of the first DNA are exposed to solvent,[13] and thus could interact with the bases of the second DNA strand without a requirement for any further structural reorganisation of the RecA filament. Linear dichroism measurements support our conclusion about the absence of any significant change in the structure of the RecA–DNA filament upon binding of the second DNA strand.[16,41]

The difference in ΔH between the binding of complementary and non-complementary DNA is rather small, of the order of 1–2 kcal/mol. A recent simulation analysis of the pairing reaction by Fulconi *et al*. showed that such a small difference favours rapid pairing of complementary DNA strands by facilitating the dissociation of mispaired DNA with partially complementary sequences.[42] It should be noted that the ΔH of binding of a complementary DNA in the RecA fibre complex is smaller than that for the formation of a poly(dT):poly(dA) duplex in the absence of RecA. Thus, RecA may allow only a relatively weak interaction between the two DNA molecules, with a correspondingly reduced activation barrier to dissociation and, in this way, facilitate a rapid search for homology.

In contrast to the binding of the second DNA, a large temperature-dependence of ΔH was observed for the binding of the first DNA strand (in the presence of ATPγS). This may be interpreted in terms of a considerable decrease in the accessible surface areas of RecA and/or DNA upon binding the first DNA strand. Several explanations could account for such a change: (1) an altered structure of DNA, such as the stretching known to occur upon binding RecA;[6] (2) change in subunit–subunit contacts of RecA for the formation of the nucleofilament with DNA; or (3) organisation of disordered parts of RecA. The first explanation appears insufficient alone, because ΔC_p depends only slightly upon the type (base-stacking state) of oligonucleotide. The second explanation may not be sufficient either, because RecA forms filamentous aggregates in the absence of DNA,[7] and the filament structure depends only to a minor extent on the presence of DNA.[43] Under the present experimental conditions, the average size of RecA oligomers is larger than a 30-mer.[7] Simply increasing the size of the filament upon DNA binding should, therefore, not affect ΔC_p so much. In fact, the ΔH of formation of the DNA–RecA complex filament without ATPγS was almost independent of temperature. Changes in the RecA–RecA interface upon DNA binding are probably not very large compared to the change expected upon binding ATP.[44] The helical pitch of a pure RecA filament (without DNA but with ATPγS) is about 9 nm.[43] This pitch is significantly larger than that of RecA without nucleotide (7 nm) and comparable to that of the complex with ATPγS and DNA (9.5 nm). Thus, the most probable explanation is the organisation of disordered parts of RecA upon binding the first DNA strand. This conclusion is supported by the observed change in the CD signal of RecA protein upon DNA binding.[33] It has been reported that a separate peptide chain of 20 amino acid residues derived from the L2 loop of RecA, which is involved in DNA binding, undergoes a structural change from random to β-pleated sheet upon DNA binding.[29] Such a change could occur in the native RecA protein upon DNA binding, although the CD change observed could not be fit by any simple change between canonical secondary structures.[33] Some protein–DNA contact might affect the CD signal. In contrast to the binding in the presence of ATPγS, the ΔC_p for binding without nucleotide cofactor was very small. Hence, ATP is necessary for the structural changes of RecA. ATP is essential for the activation of RecA: it is required for the elongation of DNA, for the immobilisation of DNA bases and for the binding of the second DNA. Thus, the structural changes of RecA indicated by our ITC measurements may be related to the activation of RecA.

The observations that ΔCp is smaller at lower pH and that protonation is required for DNA binding could indicate that RecA undergoes some conformational change upon proton uptake, and that the lower-pH form is the active one for DNA binding. The modest electrostatic effect of protonation seems unlikely as the only enhancer of DNA binding, and indeed the CD measurements support the conclu-

sion that a significant conformational change occurs in RecA upon protonation. We note that each RecA protein unit seems to require, on average, about 0.5 proton for the binding of the first DNA strand. This indicates that one proton is taken up per binding of two RecA subunits. A dimer form could very well be a functional unit of RecA, although the RecA filament is organised in a head-to-tail arrangement of monomer subunits.[7,45] A recent crystallographic analysis of the RecA homologue ScRad51 indicates that a dimer is a structural unit of the Rad51 filament.[46]

The ΔH of interaction depends clearly upon DNA sequence, and is smaller in amplitude for DNA with strong base-stacking. Nucleobases of oligo(dA), to which binding of RecA is less favoured, exhibit strong adenine-adenine stacking.[36] The smaller ΔH for double-stranded DNA could be explained by its more extensive state of stacking. RecA may require a certain amount of free energy to unstack the DNA bases for its interaction. The observation that the ΔH of the binding of the second DNA strand was smaller for DNA with a strong base-stacking tendency could thus indicate that the bases of the second DNA strand are unstacked too. In fact, the binding stoichiometry for the second DNA is about three bases (3 bp for double-stranded DNA)/RecA molecule, just as for the first DNA strand.[14] Since the helical structure of the RecA filament is virtually unaffected by the binding of a second DNA strand,[47] the bases of the second DNA strand have to be unstacked as well to fit to the stretched first DNA strand in the filament.

Our results support the proposition that RecA modifies the structure of DNA for the recognition of homologous DNA by facilitating direct base–base contacts between complementary DNA strands in its filament.

Materials and Methods

Protein and oligonucleotides

RecA was prepared as described.[48] Oligonucleotides of 36 bases were provided by Genset and used without further purification. The concentrations were determined by measuring UV absorption using the following absorption coefficients: $\varepsilon_{280} = 2.17 \times 10^4\ M^{-1}\ cm^{-1}$ for RecA, $\varepsilon_{264} = 8.5 \times 10^3\ M^{-1}\ cm^{-1}$ for oligo(dT), $\varepsilon_{257} = 8.6 \times 10^3\ M^{-1}\ cm^{-1}$ for oligo(dA) and $\varepsilon_{256} = 9.2 \times 10^3\ M^{-1}\ cm^{-1}$ for oligo(dG-dT). The absorption coefficients for the oligonucleotides were determined from their UV absorption after digestion to mononucleotides by incubation for 24 h with a mixture of nucleases (DNase I from pancreas and phosphodiesterase I from cobra venom, Sigma) and expressed in base units. ATPγS was from Boehringer Mannheim.

Isothermal calorimetry

The measurements were performed mainly with a Microcal Omega ITC apparatus, which was thermostatically controlled at 5 deg.C below the experimental temperatures with the help of a NesLab RTE 111 circulating waterbath. Some experiments were carried out in a Microcal VP-ITC apparatus. Concentrated oligonucleotide solution (500 μM in bases) was injected stepwise (in general 2.5 μl per injection) into 2 μM RecA containing 80 μM ATPγS. The duration of measurement for each injection was 300 s. The injection syringe was rotated at 400 rpm and served as a stirrer. The ΔH due to dilution of DNA was corrected. All solutions were degassed extensively by stirring under vacuum immediately before measurement. The measurements were usually performed in 10 mM potassium buffer with 1 mM $MgCl_2$, and the pH was adjusted to 6.2 or 7.4 with NaOH. When Tris–HCl or cacodylate buffer was used in place of phosphate, the concentration was 20 mM.

Circular dichroism measurements

CD was measured with a Jasco J-810 spectropolarimeter using a quartz cell with a pathlength of 1 cm or 0.2 cm (Hellma, Germany). The temperature of the cell was controlled by a programmable Peltier effect device. The spectra were measured at 20 °C in step mode (data pitch, 0.1 nm; bandwidth,2 nm; response time, 0.25 s) and averaged over three scans. Thermal denaturation of RecA was monitored by a change in CD signal at 222 nm (bandwidth, 10 nm; response time, 1 s) with a temperature increase of 1 deg.C/min. The measurements were made with continuous stirring of the sample solution to ensure temperature homogeneity.

Acknowledgements

We thank Professor Torleif Hard and Dr Kenneth Olesen (Goteborg University) for use of the VP-ITC system. This work was supported by grants from the European Community (BioMed 96–0848), the Association pour la Recherche sur le Cancer (no. 9364 and no. 3862) and the Ligue Nationale contre le Cancer, and by the Fondation pour la Recherche Médicale, the Region des Pays de la Loire, the Centre National de la Recherche Scientifique and the Swedish Cancer Foundation. F.M. was supported by a fellowship from the European Molecular Biology Organisation and T.S. was supported by a fellowship from the French Academy of Medicine.

References

1. Lusetti, S. L. & Cox, M. M. (2002). The bacterial RecA protein and the recombinational DNA repair of stalled replication forks. *Annu. Rev. Biochem.* **71**, 71–100.
2. Volodin, A. A., Voloshin, O. N. & Camerini-Otero, R. D. (2005). Homologous recombination and RecA protein: towards a new generation of tools for genome manipulations. *Trends. Biotechnol.* **23**, 97–102.
3. Little, J. W., Edmiston, S. H., Pacelli, L. Z. & Mount, D. W. (1980). Cleavage of the *Escherichia coli lexA* protein by the recA protease. *Proc. Natl Acad. Sci. USA*, **77**, 3225–3229.
4. Shibata, T., DasGupta, C., Cunningham, R. P. & Radding, C. M. (1979). Purified *Escherichia coli* RecA protein catalyzes homologous pairing of superhelical

DNA and single-stranded fragments. *Proc. Natl Acad. Sci. USA*, **76**, 1638–1642.
5. McEntee, K., Weinstock, G. M. & Lehman, I. R. (1979). Initiation of general recombination catalyzed *in vitro* by the *recA* protein of *Escherichia coli*. *Proc. Natl Acad. Sci. USA*, **76**, 2615–2618.
6. Stasiak, A., DiCapua, E. & Koller, Th. (1981). Elongation of duplex DNA by recA protein. *J. Mol. Biol.* **151**, 557–564.
7. Takahashi, M. (1989). Analysis of DNA-RecA protein interactions involving the protein self-association reaction. *J. Biol. Chem.* **264**, 288–295.
8. Bazemore, L. R., Takahahsi, M. & Radding, C. M. (1997). Kinetic analysis of pairing and strand exchange catalyzed by RecA: detection by fluorescence energy transfer. *J. Biol. Chem.* **272**, 14672–14682.
9. Bazemore, L. R., Folta-Stogniew, E., Takahashi, M. & Radding, C. M. (1997). RecA tests homology at both pairing and strand exchange. *Proc. Natl Acad. Sci. USA*, **94**, 11863–11868.
10. Ogawa, T., Yu, X., Shinohara, A. & Egelman, E. (1993). Similarity of the yeast RAD51 filament to the bacterial RecA filament. *Science*, **259**, 1896–1899.
11. Benson, F. E., Stasiak, A. & West, S. C. (1994). Purification and characterization of the human Rad51 protein, an analogue of *E. coli* RecA. *EMBO J.* **13**, 5764–5771.
12. Ellouze, C., Kim, H.-K., Maeshima, K., Tuite, E., Morimatsu, K., Horii, T. *et al.* (1997). Nucleotide cofactor dependent structural change of *Xenopus* Rad51 protein filament detected by small-angle neutron scattering measurements in solution. *Biochemistry*, **36**, 13524–13529.
13. Wittung, P., Nordén, B., Kim, S.-K. & Takahashi, M. (1994). Interactions between DNA molecules bound to RecA filament: effects of base complementarity. *J. Biol. Chem.* **269**, 5799–5803.
14. Takahashi, M., Kubista, M. & Nordén, B. (1989). Binding stoichiometry and structure of RecA–DNA complexes studied by flow linear dichroism and fluorescence spectroscopy: evidence for multiple heterogeneous DNA coordination. *J. Mol. Biol.* **205**, 137–147.
15. Chabbert, M., Lami, H. & Takahashi, M. (1991). Cofactor induced orientation of the DNA bases in single-stranded DNA, complexed with RecA protein: a fluorescence anisotropy and time-decay study. *J. Biol. Chem.* **266**, 5395–5400.
16. Nordén, B., Wittung-Stafshede, P., Ellouze, C., Kim, H.-K., Mortensen, K. & Takahashi, M. (1998). Base orientation of second DNA in the RecA-DNA filaments: analysis by combination of linear dichroism and small-angle neutron scattering in flow oriented solution. *J. Biol. Chem.* **273**, 15682–15686.
17. Nishinaka, T., Ito, Y., Kurumizaka, H., Terada, T., Yokoyama, S. & Shibata, T. (1997). An extended DNA structure through deoxyribose-base stacking induced by RecA protein. *Proc. Natl Acad. Sci. USA*, **94**, 6623–6628.
18. Nishinaka, T., Shinohara, A., Ito, Y., Yokoyama, S. & Shibata, T. (1998). Base pair switching by interconversion of sugar puckers in DNA extended by proteins of RecA-family: a model for homology search in homologous genetic recombination. *Proc. Natl Acad. Sci. USA*, **95**, 11071–11076.
19. Prévost, C. & Takahashi, M. (2004). Geometry of the DNA strands within the RecA nucleofilament: role in homologous recombination. *Quart. Rev. Biophys.* **36**, 1–25.
20. Freire, E., Mayorga, O. L. & Straume, M. (1990). Isothermal titration. *Anal. Chem.* **62**, 950A–959A.
21. Fisher, H. F. & Singh, N. (1995). Calorimetric methods for interpreting protein-ligand interactions. *Methods Enzymol.* **259**, 194–221.
22. Wittung, P., Ellouze, C., Maraboeuf, F., Nordén, B. & Takahashi, M. (1997). Thermochemical and kinetic evidence of nucelobase dependent RecA-DNA interactions. *Eur. J. Biochem.* **245**, 715–719.
23. Spolar, R. S. & Record, M. T., Jr (1994). Coupling of local folding to site-specific binding of proteins to DNA. *Science*, **263**, 777–785.
24. Leder, L., Berger, C., Bornhauser, S., Wendt, H., Ackermann, F., Jelesarov, I. & Bosshard, H. R. (1995). Spectroscopic, calorimetric, and kinetic demonstration of conformational adaptation in peptide-antibody recognition. *Biochemistry*, **34**, 16509–16518.
25. Wang, Y. & Adzuma, K. (1996). Differential proximity probing of two DNA binding sites in the *Escherichia coli* recA protein using photo-cross-linking methods. *Biochemistry*, **35**, 3563–3571.
26. Malkov, V. A. & Camerini-Otero, R. D. (1995). Photocross-links between single-stranded DNA and *Escherichia coli* RecA protein map to loops L1 (amino acid residues 157–164) and L2 (amino acid residues 195–209). *J. Biol. Chem.* **270**, 30230–30233.
27. Morimatsu, K. & Horii, T. (1995). The DNA binding-site of the RecA protein: photochemical cross-linking of Tyr103 to single-stranded DNA. *Eur. J. Biochem.* **228**, 772–778.
28. Takahashi, M., Maraboeuf, F. & Nordén, B. (1996). Locations of functional domains in the RecA protein: overlap of domains and regulation of activities. *Eur. J. Biochem.* **242**, 20–28.
29. Wang, L., Voloshin, O. N., Stasiak, A., Stasiak, A. & Camerini-Otero, R. D. (1998). Homologous DNA pairing domain peptides of RecA protein: intrinsic propensity to form beta-structures and filaments. *J. Mol. Biol.* **277**, 1–11.
30. Muller, B., Koller, T. & Stasiak, A. (1990). Characterization of the DNA binding activity of stable RecA–DNA complexes. *J. Mol. Biol.* **212**, 97–112.
31. Menetski, J. P., Bear, D. G. & Kowalczykowski, S. C. (1990). Stable DNA heteroduplex formation catalyzed by the *Escherichia coli* RecA protein in the absence of ATP hydrolysis. *Proc. Natl Acad. Sci. USA*, **87**, 21–25.
32. Datta, K. & LiCata, V. J. (2003). Thermodynamics of the binding of Thermus aquqticus DNA polymerase to primed-template DNA. *Nucl. Acids Res.* **31**, 5590–5597.
33. Wittung, P., Nordén, B. & Takahashi, M. (1995). Secondary structure of RecA in solution: effects of cofactor, DNA and ionic conditions. *Eur. J. Biochem.* **228**, 149–154.
34. Tracy, R. B. & Kowalczykowski, S. C. (1996). In vitro selection of preferred DNA pairing sequences by the *Escherichia coli* RecA protein. *Genes Develop.* **10**, 1890–1903.
35. Bar-Ziv, R. & Libchaber, A. (2001). Effects of DNA sequence and structure on binding of RecA to single-stranded DNA. *Proc. Natl Acad. Sci. USA*, **98**, 9068–9073.
36. Ts'o, P. O., Rapaport, S. A. & Bollum, F. J. (1966). A comparative study of polydeoxyribonucleotides and polyribonucleotides by optical rotatory dispersion. *Biochemistry*, **5**, 4153–4170.
37. Ferrari, M. E. & Lohman, T. M. (1994). Apparent heat capacity change accompanying a non-specific protein-DNA interaction. *Escherichia coli* SSB tetramer

binding to oligodeoxyadenylate. *Biochemistry*, **33**, 12896–12910.
38. Chabbert, M., Cazenave, C. & Hélène, C. (1987). Kinetic studies of recA protein binding to a fluorescent single-stranded polynucleotide. *Biochemistry*, **26**, 2218–2225.
39. Morrical, S. W. & Cox, M. M. (1985). Light scattering studies of the recA protein of *Escherichia coli*: relationship between free recA filaments and the recA-ssDNA complex. *Biochemistry*, **24**, 760–767.
40. Jelesarov, I. & Bosshard, H. R. (1994). Thermodynamics of ferredoxin binding to ferredoxin: $NADP^+$ reductase and the role of water at the complex interface. *Biochemistry*, **33**, 13321–13328.
41. Morimatsu, M. & Takahashi, M. (2006). Structural analysis of RecA protein-DNA complexes by fluorescence-detected linear dichroism: Absence of structural change of filament for pairing of complementary DNA strands. *Anal. Biochem.* **358**, 192–198.
42. Fulconi, R., Dutreix, M. & Viovy, J. L. (2005). Numerical investigation of sequence dependence in homologous recognition: evidence for homology traps. *Biophys. J.* **88**, 3770–3779.
43. Ellouze, C., Takahashi, M., Wittung, P., Mortensen, K., Schnarr, M. & Nordén, B. (1995). Evidence for elongation of helical pitch of the RecA filament upon ATP and ADP binding using small-angle neutron scattering. *Eur. J. Biochem.* **233**, 579–583.
44. VanLoock, M. S., Yu, X., Yang, S., Lai, A. L., Low, C., Campbell, M. J. & Egelman, E. H. (2003). ATP-mediated conformational changes in the RecA filament. *Structure*, **11**, 187–196.
45. Jonsson, M., Jacobsson, U., Takahashi, M. & Nordén, B. (1993). Orientation of large DNA during free solution electrophoresis studied by linear dichroism. *J. Chem. Soc. Faraday Trans.* **89**, 2791–2798.
46. Conway, A. B., Lynch, T. W., Zhang, Y., Fortin, G. S., Fung, C. W., Symington, L. S. & Rice, P. A. (2004). Crystal structure of a Rad51 filament. *Nature Struct. Biol.* **11**, 791–796.
47. Simonson, T., Kubista, M., Sjöback, R., Ryberg, H. & Takahashi, M. (1994). Properties of RecA-oligonucleotide complexes. *J. Mol. Recogn.* **7**, 199–206.
48. Takahashi, M. & Hagmar, P. (1991). Use of ion exchange chromatography for the study of RecA-DNA interactions. *FEBS Letters*, **279**, 270–272.

Edited by D. E. Draper

(Received 15 August 2006; received in revised form 9 October 2006; accepted 11 October 2006)
Available online 17 October 2006