Correlation of thermodynamic and genetic properties in the Tn10 encoded TET gene control region

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ABSTRACT

The thermal stability of the Tn10 encoded tetracycline resistance (TET) gene control region is investigated by melting studies using purified DNA restriction fragments containing various amounts of flanking sequences. In order to study the thermodynamic properties of this control region under conditions, where enough flanking DNA is present to mimic the situation in the chromosome, the five step melting process of a 1450-bp DNA fragment is analyzed. Because most of the sequence of this DNA is not known, the assignment of the melting transitions to segments of the DNA is done by an experimental method. This employs the preparation of subfragments from the 1450-bp DNA and comparison of their denaturation profiles with the one of the intact sequence. This approach results in the complete assignment of the five denaturation steps. Rather than from the ends, the unwinding starts from the TET gene control region in the middle of the 1450-bp sequence. A clear correlation between the thermodynamic and genetic properties of this DNA is observed. The regulatory sequence forms a small cooperative unit with the lowest stability in the entire fragment. The thermal denaturation of the TET repressor-TET operator complex reveals, that the TET repressor specifically recognizes the double stranded TET operator DNA and stabilizes this structure by 2.4°C. This result is also discussed as an example of the possible action of denaturing or stabilizing proteins on this genetic control region.

INTRODUCTION

Thermal denaturation studies on small homogeneous DNA fragments from the E. coli lactose genetic control region revealed, that the stability of this regulatory sequence is correlated to the functional domain [1,2]. Both binding sites for the cyclic AMP receptor protein (CAP) and the E. coli RNA polymerase are located within the same cooperatively melting region of the DNA. Each binding site is located close to one boundary of this 80 base pair (bp) sequence [1,2]. This result implies that a potential influence of one of these proteins on the stability of the
double helix is extended to the respective other binding site.

It was noted previously that promotors tend to occur in AT rich regions of the genomic DNA [3,4] which is consistent with the ability of RNA polymerase to unwind about ten base pairs in order to allow the synthesis of a mRNA from the DNA template [5]. Therefore, it is interesting to study the thermal stability of other regulatory DNA sequences in an attempt to find correlations with the biological function. Recently, we reported the isolation of the molecular components involved in the regulation of the tetracycline resistance mediating (TET) gene located on the transposon Tn10 [6]. In this article we analyze the thermal stability of this DNA sequence and interpret it with respect to its genetic loci. In order to study the TET operator-promotor region we use a 1450-bp long DNA sequence to avoid end effects. As a result of the high molecular weight the denaturation profile is multiphasic which hampers a straightforward assignment of the subtransitions to the sequence of the DNA.

Several theoretical attempts have been made to assign melting transitions in multiphasic denaturation profiles to specific segments of the DNA [7,8,9]. Any of these procedures requires knowledge of the sequence of the DNA fragment studied. An experimental approach to the assignment problem is to prepare restriction fragments of a high molecular weight DNA and compare the melting of these small molecules with that of the intact DNA. Studies using this approach were performed for phage ØX174 RF and fd RF DNA [10,11]. Due to their molecular weight, this method was not sufficient to interpret the melting of these molecules in detail.

Because a major part of the sequence surrounding the TET gene control region is not known we chose the experimental approach. The study of six subfragments results in the assignment of the five step melting of the 1450-bp DNA fragment. The result of this determination is proven by a specific stabilization of the TET operator region in the presence of the TET repressor.

MATERIALS AND METHODS

General Methods

Plasmid preparation was essentially done as described [12].
The plasmids used to prepare the respective DNA fragments were pRT29 [13], pWH106, pWH141, and pWH122 [6]. All restriction endonucleases were purchased from BRL, Bethesda Md, with the exception of Eco RI which was prepared from E. coli RY13 by a published procedure [14]. After restriction enzyme digestion the resulting DNA mixtures were separated using RPC-5 chromatography as described [12,15].

Preparation of the DNA samples

The purified restriction fragments were dialyzed once versus 2.5 M NaCl, 5 mM Na-cacodylate, pH 7.0, 0.1 mM EDTA and then four times versus 5 mM Na-cacodylate, pH 7.0, 0.1 mM EDTA. About 0.1 A₀ units were then mixed with the appropriate amount of NaCl stock solution containing 5 mM Na-cacodylate, pH 7.0, 0.1 mM EDTA to yield the desired final ionic strength indicated in the legends to the respective figures. The total volume of the sample was 0.3 ml. The ionic strength of the stock solutions was checked by their conductivity using a model LF410 meter from WTW, Weilheim, Germany. The mixture was centrifuged to remove dust particles, placed in a 1 ml jacketed quartz cuvette with an optical path length of 1 cm and saturated with helium. The cuvette was tightly stoppered, placed in the thermostatable cuvette holder and thermostated at 50°C. After thermal equilibrium was achieved, the concentration of the DNA was determined by reading the A₀ twenty-sixth.

Measurement of absorption-temperature profiles

Absorption-temperature transitions were recorded at various wavelengths using a Zeiss PMQ III spectrophotometer equipped with micro optics and deuterium lamps of selected stability. The temperature was monitored by a calibrated thermistor placed in the circulating liquid behind the cuvette. Temperature was controlled with a Haake F3C thermal bath equipped with a Haake PG10 temperature controller. A second thermal bath was used to maintain the temperature of the photomultiplier housing at 40°C. The heating rate did not exceed 0.07°C/min. Absorption-temperature data pairs were recorded using a Commodore CBM model 3016 interfaced to the Zeiss and the thermistor. In this process 50 individual absorption-temperature readings were averaged and stored. The data were then transferred to a Hewlett Packard model HP 9830 A com-

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computer for further handling. As an additional control of the measurement the absorption was recorded on chart paper.

Handling of the data

The data were processed using a HP 9830 A computer from Hewlett Packard. They were smoothed by fitting successive segments varying between 13 and 21 data points to a linear equation, from which the derivative of the segment middle was determined. It was made sure by using less rigorous smoothing procedures that the fit had no influence on the shape of the melting curve. Any other processing of the data was as described [16]. The melting curves were plotted using a HP 9862A plotter from Hewlett Packard. The number of base pairs denaturing under a subtransition in multiphasic melting curves was determined from the area of the respective subtransitions. For this purpose the DNA denaturation profiles were recorded at 272 nm to assure equal contributions from AT and GC base pairs [17]. Prior to the area analysis given in table I the θ values were corrected for single stranded unstacking by neglecting the noncooperative increase of the absorption between the subtransitions.

RESULTS

Preparation of the DNA fragments and assignment strategy

The primary source of the DNA restriction fragments used in this study is the plasmid pRT29 which contains about 2 kbp of Tn10 DNA including the TET operon [13,18]. Cleavage of this plasmid with Eco RI results in the release of a 1450-bp fragment which contains the TET operon. Figure 1 displays the 1450-bp DNA with respect to the genetic loci of the TET operon [6,13,18]. Three subfragments were prepared to study the influence of flanking DNA sequences on the thermodynamic behavior of the TET operon [18]. The respective sizes are taken from ref. 18. The assignment of individual melting transitions in the multiphasic denaturation of the 1450-bp fragment is achieved by studying the denaturation of smaller fragments derived from the action of various restriction endonucleases. As indicated in figure 1 the 1450-bp DNA can be cleaved in two parts using Xba I. The resulting fragments are 790- (left side in figure 1) and 660-bp long [13]. Cleavage with Hinc II results in three pieces
Figure 1 Biological activity and partial restriction map of the DNA fragments. The dashed lines in the upper panel denote the mRNAs for the TET repressor and the TET protein. The letter O denotes the location of the TET operator as determined by protection experiments [6]. The boxes refer to the DNA fragments with the numbers indicating their size. The smallest fragment is 187-bp long. The restriction enzymes indicated on the right side were used to obtain the original fragments [18]. The 1450-bp Eco RI fragment originates from pRT29 and contains approximately 135-bp pVH51 DNA at the leftward end. Restriction sites on the 1450-bp fragments are denoted as H = HincII, X = Xba I, T = Tag I, and S = Sau 96I.

which are, starting from the left side in figure 1, 135-, 695-, and 620-bp long. Because binding of the TET repressor to the TET operator results in protection of the middle HincII site, the reaction products of a HincII digest in the presence of the TET repressor are 135- and 1315-bp fragments [6]. The site designated S in figure 1 is cleaved by Sau 96I resulting in 1335- and 115-bp fragments [18]. The latter two reactions can therefore be used to study the influence of removing sequences from the ends on the denaturation of the 1450-bp DNA. Finally, the assignment of melting transitions in the TET protein structural gene was possible by studying a 545-bp DNA resulting from a double digest of the 1450-bp fragment with Xba I and Sau 96I.

All fragments used in this study were purified by RPC-5 chromatography following the respective restriction digest [15]. Gel analysis revealed, that no contaminating DNA fragment was present within the limits of detectability. It is estimated that contaminations if present at all, amount to less than 2% of the total DNA.

Thermodynamic stability of the TET gene control region

Figure 2 displays the melting curve of the four TET operon containing DNA fragments displayed in figure 1. All melting
Figure 2  Thermal denaturation of the TET operator containing DNA fragments. Panel A shows the thermal denaturation of the 501-bp fragment (dashed line) offset by 1.6 on the vertical axis. Panel B shows the unwinding of the 461-bp DNA (solid line) offset by 1.2 on the vertical scale. Panel C displays the denaturation of the 187-bp (dotted line) and the 1450-bp (solid line) fragments. All experiments were done at an ionic strength of 88 mM NaCl.

curves are on the same temperature scale to facilitate their comparison. The only fragment denaturing in a single cooperative transition is the 187-bp DNA which also has the lowest stability of all sequences in these fragments. Table 1 summarizes the denaturation temperatures ($T_M$) and the sizes of the denaturing DNA segments for the fragments displayed in figure 1. It is obvious that some rearrangements of the cooperatively denaturing segments occurs in dependence of the neighboring sequences. Table 1 reveals that the denaturation of the 187-bp DNA segment is also found in the 501-bp fragment. Inspection of figure 1 shows that this DNA consists of the 187-bp sequence plus 314-bp added on the left end of figure 1. This results in a sta-
Table 1  $T_m$ values and size of the DNA segments denaturing in the respective transitions

<table>
<thead>
<tr>
<th>Fragment size (bp)</th>
<th>187</th>
<th>461</th>
<th>501</th>
<th>1450</th>
<th>790</th>
<th>660</th>
<th>545</th>
</tr>
</thead>
<tbody>
<tr>
<td>$T_m$ (°C) (bp)</td>
<td>76.4</td>
<td>76.8</td>
<td>78.9</td>
<td>79.8</td>
<td>-</td>
<td>82.2</td>
<td>79.0</td>
</tr>
<tr>
<td>$T_a$ size (bp)</td>
<td>187</td>
<td>124</td>
<td>179</td>
<td>144</td>
<td>67</td>
<td>778</td>
<td>80</td>
</tr>
</tbody>
</table>

The accuracy of the $T_m$ determination is ±0.5°C

The accuracy of this determination is approximately ±15%

The data for these fragments are grouped according to their assignment.

In the 461- and 1450-bp fragments (as determined from evidence presented below) this cooperative unit appears to be about 50-bp shorter. The 461-bp DNA has 274-bp added to the right side in figure 1 whereas the 1450-bp DNA contains the 187-bp in the middle of the fragment. Therefore, the 50-bp which are removed from the cooperative transition in the 461- in comparison to the 501-bp DNA must be located on the right side of the 187-bp DNA as displayed in figure 1. The remaining 124-bp are less stable by 2.1°C than the respective part of the 501-bp DNA. This finding suggests that the 50-bp are more GC rich than the remaining 124-bp. Thus, if they are not at the end of a fragment they tend to be part of another cooperative segment.

The 1450-bp fragment denatures in five clearly resolved transitions. Comparison of its melting behaviour with the three other fragments in figure 2 suggests that the first transition involves the denaturation of the TET gene regulatory region, because that part of the other fragments is always most unstable. In contrast to the other DNAs, however, this sequence is not at
the end of the 1450-bp fragment which should result in a considerable stabilization of these base pairs. As a result of these considerations, one has to exclude that melting from one of the ends of the 1450-bp DNA gives rise to the first transition as opposed to the TET regulatory sequence.

Assignment of the sequences melting under the five subtransitions of the 1450-bp DNA fragment

The first experiment in this series is designed to exclude contributions from sequences at the end of the 1450-bp DNA to the first transition in the melting curve. This goal is achieved by removing 135-bp and 115-bp from the left and right end of the 1450-bp DNA as drawn in figure 1. Figure 3 compares the thermal denaturation of the 1450-bp and the resulting 1315-bp and 1335-bp fragments. It may be clearly derived from figure 3 that the first subtransition remains unchanged. Subtle differences are observed as the result of shortening the 1450-bp which are

Figure 3 Influence of the end sequences on the melting of the 1450-bp DNA. Panel A displays the melting of the 1315-bp fragment which has the leftward 135-bp from the 1450-bp DNA removed, offset by 1.2 on the vertical scale. Panel B shows the unwinding of the 1335-bp DNA which lacks 115-bp from the right end of the 1450-bp sequence offset by 0.6 on the vertical scale. Panel C shows the denaturation of the 1450-bp DNA for comparison. All experiments were done at 88 mM NaCl.
difficult to interpret because the difference in length is less than 10%. The 1315-bp denaturation curve, however, seems to be altered in the main subtransition. This observation will be confirmed below. The important conclusion from the data in figure 3 is that the denaturation of the 1450-bp DNA starts somewhere inside the double helix rather than at one of the ends.

Because it is shown that the first melting transition in the 1450-bp fragment involves the TET gene regulatory sequence it is of advantage for the further assignment to cut the 1450-bp DNA in this area. This should not influence the subtransitions in either part of the molecule because their neighboring sequences are in the same helical state as in the complete 1450-bp fragment. Figure 4 compares the melting areas for these two half molecules with the one of the intact 1450-bp sequence. The two halves were generated by Xba I cleavage (compare figure 1). The resulting left part of the DNA is 790-bp and the right half is 660-bp long. Comparison of the melting curves in figure 4 reveals rather clearly that the main transition in the 1450-bp denaturation results from the unwinding of the approximately 760-bp from the left end of the molecule. The three subtransitions at higher temperatures are caused by sequences in the right part as indicated by the 660-bp fragment melting curve. The number of bp melting under the subtransitions along with the T_M values are summarized in table 1. It is clear that the denaturation profiles of the subfragments agree quantitatively with the one of the 1450-bp DNA. This may be taken as an additional indication for the fact that the Xba I site used to generate these fragments is located within the first melting transition of the 1450-bp DNA.

Assignment of the last three subtransitions succeeds by making use of the Sau 96I restriction site (compare figure 1). Figure 5 compares the denaturation curves of the 660-bp Xba I - Eco RI right half of the molecule with the 545-bp Xba I - Sau 96I subfragment [18]. It is rather clear that the subtransition just below 83°C belongs to the sequence which is removed by Sau 96I cleavage. It may, therefore, be concluded that the third step in the denaturation of the 1450-bp fragment is due to the unwinding
Figure 4 Melting transitions on the left- and rightward half of the 1450-bp DNA. Panel A (dashed line) displays the thermal denaturation of the 660-bp TET gene part of the 1450-bp DNA offset by 1.6 on the vertical scale. Panel B shows the unwinding of the 790-bp TET repressor gene side of the 1450-bp DNA offset by 0.8 on the vertical scale. Panel C gives the 1450-bp melting curve for comparison. All experiments were performed at 88 mM NaCl.

of approximately 160-bp from the right end of the molecule as drawn in figure 1.

The last two steps are resolved by comparing the denaturation of the 461-bp Alu I with the 545-bp Xba I - Sau 96I fragment. The 461-bp DNA lacks 122-bp from the Sau 96I side of the 545-bp DNA and contains additional 38-bp on the Xba I side. The melting curve as shown in figure 2 is redrawn in figure 5 to allow a better comparison. The two denaturation steps around 85°C in the 545-bp DNA are effected by the lack of the 122-bp in the 461-bp DNA, which shows a single cooperative transition at a temperature close to 84°C.
Figure 5  Melting of subfragments from the right half of the 1450-bp DNA. Panel A displays the unwinding of the 660-bp sequence from the right side of the 1450-bp DNA offset by 0.6 on the vertical scale. Panel B shows the melting of the 545-bp DNA lacking the right-most 115-bp from the 660-bp fragment offset by 1.2 on the vertical scale. Panel C (dashed line) displays the denaturation of the 461-bp fragment (compare figure 1). All experiments were performed at 88 mM NaCl.

This result was confirmed by melting a mixture of the 461- and 660-bp fragments in the same experiment, which lead to identical differences in the $T_M$ values. Therefore, some rearrangement of the cooperative units must occur in this fragment compared to the 545-bp DNA. However, any possible explanation requires the loss of bp from the most stable region of the 545-bp piece to yield the 461-bp fragment. It may be concluded, that the middle transition in the 660-bp right half molecule of the 1450-bp DNA is located at the beginning of the TET gene whereas the last transition involves the following 250-bp of that gene. The $T_M$ values and area analyses of these denaturation experiments are listed in Table 1. It is noteworthy that the melting enthalpies of all transitions support the area analysis of the
melting experiments with the exception of the second sharp transition in the 1450-bp DNA [16,19]. It may thus be, that this transition is the result of two melting processes separated only by a small difference in $T_m$.

The result of this chemical approach to assign subtransitions in a thermal denaturation experiment to particular sequences of the denaturing molecule is the complete reaction scheme for the unwinding of the 1450-bp DNA shown in figure 6. The location of the TET gene regulatory sequences is also given in figure 6. Within this sequence of 1450-bp the operator-promotor region of this bidirectional control element is the least stable region; it is even less stable than sequences at the ends of this fragment. In the second step the complete structural gene for the TET repressor denatures followed by several tran-

![Figure 6](image_url)
Figure 7 Effect of TET repressor on the denaturation of the 1450-bp DNA. Panel A displays the melting of the 1450-bp DNA in the presence of a fourfold molar excess of TET repressor [6] offset by 1.0 on the vertical scale. Panel B shows the 1450-bp denaturation curve for comparison. The ionic strength in these experiments was 4 mM NaCl.

Influence of the TET repressor on the denaturation of the TET operator

Because the TET operator region is a clearly separated small cooperatively denaturing unit in the 1450-bp DNA we evaluated the influence of TET repressor-TET operator complex formation on the thermal stability of the operator sequence. In order to shift the $T_M$ of this sequence below the denaturation temperature of the TET repressor protein the experiment was run at an ionic strength of 4 mM NaCl [6]. Figure 7 compares the melting of the 1450-bp DNA alone with the thermal transition curve obtained in the presence of stoichiometric amounts of TET repressor which forms a tetrameric complex with the TET operator [6]. Next to a slight shift of the $T_M$ values which probably arises from an in-
crease in ionic strength due to the addition of TET repressor solution, the denaturation of the TET operator sequence is stabilized specifically by 2.6°C. As a result it denatures at a higher temperature than the main transition in the 1450-bp melt. This finding confirms the assignment in figure 6 and indicates that the TET repressor recognizes the double stranded TET operator and stabilizes it against thermal denaturation.

DISCUSSION

The goal of this study is to determine possible correlations of the TET regulatory sequence with the thermodynamic properties of this DNA region. Comparison of the thermal denaturation curves of the 187-, 461-, 501-, and 1450-bp DNA fragments in figure 2 reveals that both the thermal stability and the size of the cooperatively melting segment which includes the TET operator-promoter region depend on the environment. In the 187- and 501-bp fragments the regulatory sequence is part of an approximately 180-bp long cooperatively unwinding segment, whereas in the 461- and 1450-bp fragments the cooperatively melting unit is only approximately 140-bp long. In order to derive results reflecting the situation of this operator-promoter segment as similar as possible to the chromosomal state, we studied the thermodynamic properties of the 1450-bp DNA. Figure 1 indicates that this DNA contains the TET gene regulatory region almost in the middle with approximately 800-bp flanking the TET repressor gene side and roughly 600-bp flanking the TET gene side. Figure 2 reveals that this molecule denatures in five resolved steps which creates an assignment problem. This difficulty is solved by the preparation of subfragments from the 1450-bp sequence. The comparison of the melting processes for six different subfragments with the one of the 1450-bp DNA leads to a complete assignment of the five melting transitions to the unwinding of specific segments in the 1450-bp sequence. This approach is the only possible assignment strategy in this case, because only a small portion of the DNA sequence is known (Wray and Reznikoff, personal communication; Postle and Bertrand, personal communication).

The denaturation map shown in figure 6 reveals that the bi-
directional TET operator-promoter system consisting of two overlapping promoters and possibly two operators [18] is the most unstable part of the 1450-bp sequence. This result agrees well with the notion that promoters tend to occur in AT rich regions of genomic DNA [3,4,20]. It is well established that E. coli RNA polymerase unwinds about ten base pairs upstream of the mRNA start nucleotide upon binding to a promoter [5]. The thermodynamic property of the 1450-bp fragment is such, that the RNA polymerase binding site requires the smallest energy for this process on the entire fragment. It is also noteworthy that the cooperative unit consisting of the operator-promoter sequence is only about 140-bp long. Therefore, the TET gene control region can be influenced specifically by either stabilizing or destabilizing ligands. A similar agreement of regulatory with thermodynamic properties has been found for the E. coli lactose genetic control region [1,2].

It has been observed in minicells that the TET repressor gene may be expressed at a higher level than the TET gene [18]. This result may also be correlated to the thermodynamics of these structural genes. Figure 6 reveals that the TET repressor gene is less stable than the TET gene. The decreased stability may result in an increased rate of transcription by facilitating the unwinding activity of RNA polymerase.

The possible specific effect of a ligand on the TET gene regulatory region is demonstrated by the melting experiment employing a TET operator-TET repressor complex. As anticipated the presence of the TET repressor results in a shifted T_M of the TET operator sequence which reflects a significant, specific stabilization of this region upon complex formation (compare figure 7). This result indicates that the TET repressor recognizes the double stranded structure of the TET operator and counteracts the RNA-polymerase action by stabilizing this sequence.

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