ELSEVIER

Biophysical Chemistry 81 (1999) 207–221

Biophysical Chemistry

www.elsevier.nl/locate/bpc

Comparison of the thermal denaturation behaviour of DNA-solutions and metaphase chromosome preparations in suspension

D. Wolf, J. Rauch, M. Hausmann[1], C. Cremer*

Applied Optics and Information Processing, Institute of Applied Physics, Ruprecht-Karls-University, Albert-Ueberle-Str. 3-5, D-69120 Heidelberg, Germany

Received 16 July 1998; received in revised form 23 July 1999; accepted 23 July 1999

Abstract

Hyperchromicity measurements are well established to analyse the thermal denaturation behaviour of pure DNA sequences in solution. Here, we show that under appropriate experimental conditions this technique can also be applied to study thermally controlled conformation changes of higher order DNA–protein complexes as for instance metaphase chromosome preparations in suspension. A computer controlled sensitive, upright double beam photometer with a heatable cuvette was constructed. Measurements of the temperature dependent extinction of both, solutions and particle suspensions are possible, since sedimentation effects of particles can be neglected due to the vertical optical axis in the probe cuvette. Thermal denaturation of metaphase chromosome preparations of human and Chinese hamster cells was investigated and compared to melting profiles of DNA solutions for two excitation wavelengths, 256 and 313 nm. The influence of neutral and low pH was considered. The results indicate that metaphase chromosome preparations show a thermal denaturation behaviour different from pure DNA. Whereas DNA solutions showed one pH dependent melting peak at 256 nm only, the peak pattern of metaphase chromosome preparations showed a large variability both at 256 and 313 nm. At neutral pH, in two temperature regions (40–55°C and 75–82°C) peaks were found indicating chromosome typical conformation changes independently from the mammalian cell species (Chinese hamster, human). In contrast to pure DNA, no typical reduction in the temperatures of peak maxima with decreasing pH was found for metaphase chromosome preparations of both cell types.

* Corresponding author. Tel.: +49-6221-549250; fax: +49-6221-549262.
E-mail address: cremer@popeye.aphys2.uni-heidelberg.de (C. Cremer)
[1] Present address: Institute of Molecular Biotechnology, Jena, Germany

PII: S0301-4622(99)00096-4

These results may be relevant for further systematic studies of efficient thermal probe/target denaturation procedures in non enzymatic DNA–chromosome in situ hybridisation.

Keywords: Thermal denaturation; DNA; Metaphase chromosome preparations; Extinction measurements

1. Introduction

A variety of physical properties of pure DNA are changed characteristically by chemical (e.g. by acids or lyes or solvents) and/or heat treatment over a critical temperature. These effects are mostly due to strand separation of the DNA double strand accompanied by helix-to-helix and helix-to-coil transitions. This thermally induced strand separation (also called 'DNA denaturation' or 'DNA melting') depends on the in situ chemical conditions in the solution, for instance the amount of mono- or divalent positive ions, and the presence of ligand formers [1–5].

DNA melting is usually measured photometrically by the temperature depending extinction at 260 nm [6–8]. The transition probability of the π-electrons of the DNA bases corresponding to the absorption is approximately 30% higher for single stranded DNA than for double stranded. Thus, DNA melting shows a typical step-like hyperchromicity [2,8]. The melting-point is defined by the point of inflection of the hyperchromicity curve where 50% double-stranded and 50% single-stranded DNA coexist. This corresponds to a peak maximum in the first derivative of the hyperchromicity curve. In the following this peak profile is called melting curve or denaturation profile, respectively.

DNA from genomic origin is composed of different repetitive (AT-rich) and single copy (GC-rich) sequences which show several different melting points. In addition, molecules affecting DNA stability influence this melting behaviour. The influence of substances which act in solution and which do not significantly interfere with the tertiary structure of the DNA macromoelcule is well understood. Here, shifts in the peak pattern of the melting curve can mainly be interpreted by a thermodynamic description of binding with respect to the electrostatic near-field surrounding of the negatively charged DNA-backbone.

However, the influence of organic partners such as proteins cannot be described on solvent-effect parameters, since complex forming interactions with DNA have to be considered. Experiments with chromatin for instance have shown an increased melting point accompanied by a broadening of the temperature range of hyperchromicity [8]. For specific histon–DNA complexes different regions of phase transitions were found and interpreted to indicate different states of the DNA and DNA–histon binding, respectively [9–12]. Other experiments with interphase and metaphase chromatin [13], however, suggested that higher order structures do not influence the denaturation behaviour of nucleo-proteins.

However, to draw the conclusion that thermally treated mammalian chromosomes in metaphase or interphase show an extinction behaviour very similar to pure chromatin or, moreover, to DNA appears to be oversimplified, since not only histons but also non-histone proteins may play a (still unknown) considerable role in metaphase chromosome organisation. In order to verify or falsify the hypothesis that in general higher ordered chromatin structures as they exist in interphase and metaphase chromosomes [14] have a considerable influence on the chromatin denaturation behaviour, metaphase chromosome preparations were used. They have a large amount of compactly organised chromatin (i.e. in the chromosomes) which would in case of a falsification of the hypothesis increase the reliability of the investigation.

The assumption that DNA and chromosomes are different concerning their denaturation behaviour was supported by results of the thermally dependent quantum yield of the fluorochrome

Hoechst 33258 used for DNA and chromosome staining [15]. This approach to correlate fluorescence effects to denaturation effects has the disadvantage that temperature dependent characterstics of the fluorochrome itself, e.g. thermal stability, have to be considered carefully in order to calibrate the DNA and chromosome measurements. Therefore, it was desirable to also investigate chromosome denaturation by extinction measurements. However, in contrast to relatively small sequences of pure DNA (usually $\ll 10^9$ Da), mammalian metaphase chromosome preparations are suspensions of relatively large particles (DNA–protein complexes typically $\cong 10^{11}$ Da) subjected to sedimentation during long term (i.e. hours) measurements. To overcome spurious 'hyperchromicity effects' induced by particle sedimentation, an appropriately designed photometer with a vertical optical axis was built. Here, we want to describe the features of the system and its suitability for measurements of both DNA solutions and metaphase chromosome preparations. Extinction measurements at approximately 260 nm (DNA absorption maximum at neutral pH) and approximately 320 nm (no DNA absorption at neutral pH) were performed to obtain 'denaturation profiles' (peak pattern of the first derivative of the melting curve) of metaphase chromosome preparations of human and Chinese hamster cells under different conditions in comparison to DNA.

2. Materials and methods

2.1. Metaphase chromosome preparations

Metaphase chromosome preparations were obtained from human lymphocytes isolated from peripheral blood and fixed with methanol/acetic acid by standard techniques [16,17]. To prepare a suspension that can be heated up to 95°C, the metaphase preparation was centrifugated and gently resuspended in 1× SSC buffer (0.15 M NaCl, 0.015 M sodium citrate) at pH 7.0.

For other experiments, cells of the Chinese hamster cell line DON and the human cell line NC37 (Caucasian male) were used. Mitotic cells were synchronised by a colcemid block of 4 h (0.25 μg colcemid/ml medium). The cells were harvested in mitosis by shaking off and pelleted by centrifugation (350 *g*, 15 min). After incubation for 5 min at a temperature of −20°C, the pellet was resuspended in a hypotonic solution (10 mM Tris–HCl, 5 mM $MgCl_2$, 10 mM NaCl, pH 7.5) and incubated again for 15 min at room temperature. The hypotonic solution was removed by centrifugation (350 *g*, 10 min), and 1 ml isolation buffer TAcCaM [15] (25 mM Tris–acetic acid, 5 mM $CaCl_2$, 5 mM $MgCl_2$, pH 3.2) was added. In this isolation buffer, the mitotic cells were sonicated in a water bath to disrupt the cell membrane and to obtain a suspension with dispersed chromosomes. Before the measurements, the suspension was again centrifuged and transferred to 1 × SSC.

In cases where hexandiol was used in the measurement it was added to the isolation buffer up to a final concentration of 750 mM [18] before sonication. 200–300 μl of the metaphase chromosome preparations were diluted to a final concentration of 20 μg chromosomes/ml suspension.

2.2. DNA solutions

For comparison, two different types of DNA were used:

1. Human DNA was extracted from lymphocyte metaphase chromosome preparations by protease digestion and column purification. A commercially available DNA isolation kit (Quiagen-pack 100, Diagen GmbH, Hilden, Germany) was used.
2. Commercially available calf thymus DNA (CT-DNA) (type I, 'highly polymerized', Sigma GmbH, Deisenhofen, Germany) was used as a reference for Chinese hamster metaphase chromosome preparations. Due to its base composition this DNA is compatible to Chinese hamster DNA [15].

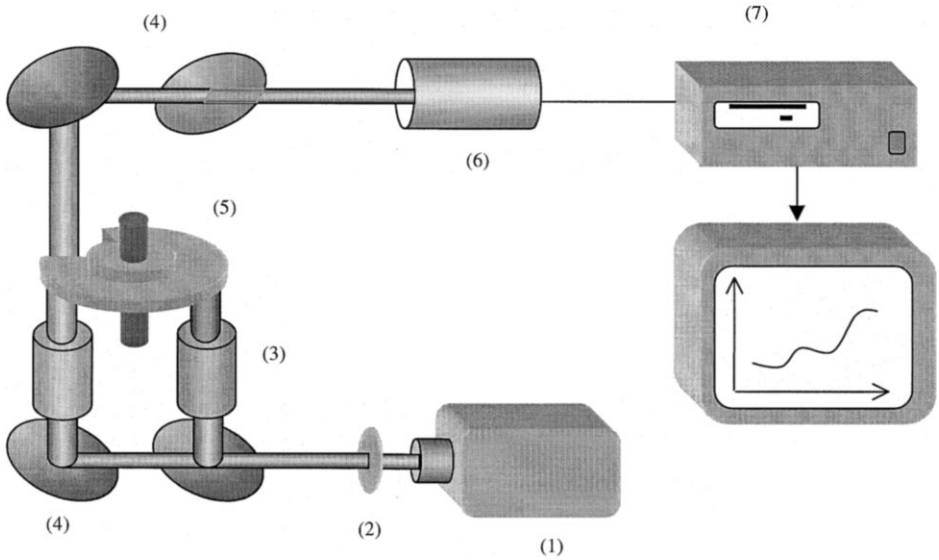
For hyperchromicity measurements, the DNA was solved in 1 × SSC or TAcCaM, respectively, at a final concentration of approximately 20 μg DNA/ml solution.

2.3. Photometer

To investigate the thermally induced absorption behaviour of both, solutions and particle suspensions, a photometer system is required that eliminates sedimentation effects in the optical signal especially during long-term (1–2 h) measurements. Therefore, a computer controlled upright, double beam photometer was built [17]. The entire device (Fig. 1) consists of three functional subunits:

1. the optical system with a specially designed, precisely heatable cuvette for probe and reference;
2. the electronic control unit for signal detection, temperature registration and heating; and
3. the data processing unit for digitisation and computing of temperature and extinction data and online control of the system.

As a light source a deuterium lamp (J02, LOT GmbH, Darmstadt, Germany) is used. The light beam is coupled vertically into the probe and reference chamber of the cuvette via a 256 nm bandpass filter or 313 nm bandpass filter, respectively. The cuvette is made of a stainless steel block ($70 \times 30 \times 12$ mm^3) with appropriate 3 mm thick quartz glass windows compensating temperature-dependent volume changes by Viton rings (vi 500). Probe and reference chamber have a volume of approximately 100 μl each. To avoid



(1) UV-lamp
(2) Filter
(3) Double Cuvette
(4) Mirrors
(5) Chopper
(6) Photomultiplier
(7) Data Processing Unit

Fig. 1. Schematic representation of the computer controlled set-up for denaturation measurements of particle suspensions.

temperature gradients within the steel block, heating elements (Philips 2NcNc 10, 300 mm) are symmetrically arranged around the cuvette. The heating voltage is computer controlled between 7 and 22 V corresponding to a maximum heating power of 130 W. The temperature of the cuvette is measured by the resistance of a platin sensor Pt 1000 (PCP 1.32/10/10, Jumo, Fulda, Germany) following a well known linear–quadratic-temperature-resistance correlation. This signal triggers the heating control. Despite this heating control, the system cannot properly be used for renaturation measurements since the device has no active cooling element to run a given cooling gradient. This means that the system is mostly useful to measure extinction changes with increasing (= denaturation), but not with decreasing (= renaturation) temperatures.

After passing the probe and reference chamber the two light beams are directed on one Peltier-cooled photomultiplier (Thorn Emi, 9635 QB) with a nominal gain of 2.5×10^7. A continuously rotating chopper wheel allows to alternately discriminate probe and reference beam. The photo current is transferred into voltage values between -5 V and $+5$ V. This signal and the temperature signal are digitised with a programmable ADC/DAC unit (DAP 1200/4, 150 kHz, Datalog, Mönchengladbach, Germany) and further treated by a completely home developed software on a personal computer.

Each individual measurement triggered by one full rotation of the chopper wheel consists of three-types of values: light sample chamber, light reference chamber, time of registration. Sixteen of these values are averaged and the mean is correlated to the respective temperature value for further evaluation.

2.4. Data processing

For data processing and graphical representation the original data are rescaled into absolute temperature values T(°C) and 'extinction' differences $\mathrm{d}(\mathrm{d}E)/\mathrm{d}T$ [OD/°C] (OD: optical density). Here, $\mathrm{d}E$ is derived from the absorption which is defined as the logarithm $\log_e$ of the ratio of the light intensity $I_{ref}(T)$ passing the reference and that passing through the sample $I_s(T)$. $\mathrm{d}E$ is defined as the difference between the absorption at the actual temperature T and the initial temperature T_o on the basis of $\log_{10}$:

$$\mathrm{d}E[OD] = \log_{10} e \cdot \left(\log_e \frac{I_{\mathrm{ref}}(T)}{I_s(T)} - \log_e \frac{I_{\mathrm{ref}}(T_o)}{I_s(T_o)} \right)$$

Assuming that concentration changes of the sample are negligible during the measurement the absorption difference $\mathrm{d}E$ can be referred as extinction difference. In order to obtain the denaturation profile as the first derivative of $\mathrm{d}E$, the data of a complete temperature interval were transferred into a continuous function by Householder transformations. This function is expanded by the linear combination of orthogonal cosinus functions

$$\cos\left(k \cdot T \cdot \frac{2\pi}{l} \right)$$

where l is the temperature interval chosen for continuous expansion (here the complete range of the measurement). T is the temperature value on the abscissa, k is the order of the expansion which can be chosen up to $k = 100$. Typically $k = 20$ was used in the measurements presented here. In the first derivative, $k = 100$ corresponds to a peak to peak resolution of 0.5°C and a peak localisation precision of 0.01°C [20]. However, this should not be mixed up with the apparative error of measurement which is ± 0.5°C.

The cosinus-series, however, requires the gradient to be zero for the boundary values. Since this cannot be usually satisfied by the measured values, the boundary regions of the measurement were modified to fulfil this condition. This may trigger additional peaks at the lowest and the highest temperature values which hence should not be considered for evaluation. [Note: $\mathrm{d}E$ as well as $\mathrm{d}(\mathrm{d}E)/\mathrm{d}T$ may also become negative. For the analysis of denaturation profiles, however, only the positions of peaks on the abscissa were relevant. In this context a peak is a local maximum of $\mathrm{d}(\mathrm{d}E)/\mathrm{d}T$ with a difference to the neighbouring local minima which is higher than the apparative resolution (see Section 2.5)].

2.5. Apparative resolution of the photometer

The electronics allows a temperature resolution of 1.5×10^{-3}°C. However, calibration and systematic errors revealed an effective temperature resolution of ± 0.5°C. The optical resolution was defined by the mean difference between a local maximum and the two neighbouring local minima of the denaturation profile $\mathrm{d}(\mathrm{d}E)/\mathrm{d}T$ vs. T. Using H_2O as sample and reference, the optical resolution was measured and estimated to be 11×10^{-4} OD for 256 nm excitation and 3.0×10^{-4} OD for 313 nm [21]. The reproducibility of the measurements was shown by the melting point

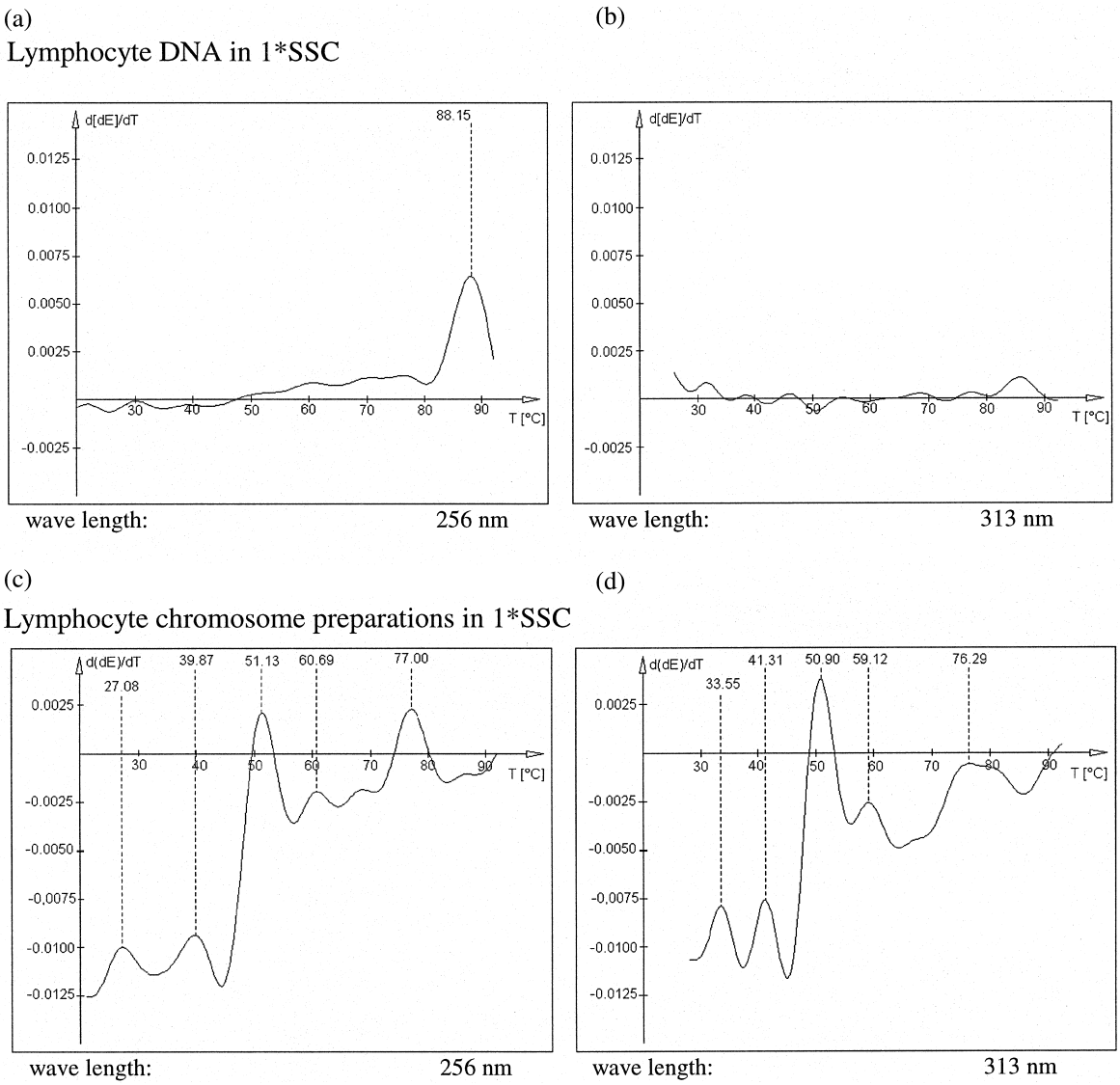


Fig. 2. Melting curves (first derivative of the extinction function) $\mathrm{d}(\mathrm{d}E)/\mathrm{d}T$ vs. temperature T of: (a) isolated human lymphocyte DNA in $1 \times$ SSC at 256 nm; and (b) at 313 nm. For metaphase chromosome preparations of human lymphocytes in $1 \times$ SSC the denaturation profiles are shown in: (c) at 256 nm; and (d) at 313 nm. Note: The small fluctuations in Fig. 2b are effects due to the cosinus expansion.

data obtained for a series of seven measurements of calf-thymus (CT) DNA in $0.1 \times$ SSC. In all cases, the melting point was within (73.7 ± 0.5) °C and thus within the apparative accuracy [21].

2.6. UV-spectrometer

For some control measurements, a PERKIN ELMER Lambda 14 UV/vis spectrometer with a heatable cuvette was used. It allows double beam measurements from 190 to 1100 nm at a given temperature. In contrast to the photometer developed here (see Section 2.5) and described above, this commercial instrument is unable to run in a temperature gradient mode. In addition, it has a horizontal optical axis through the specimen cu-

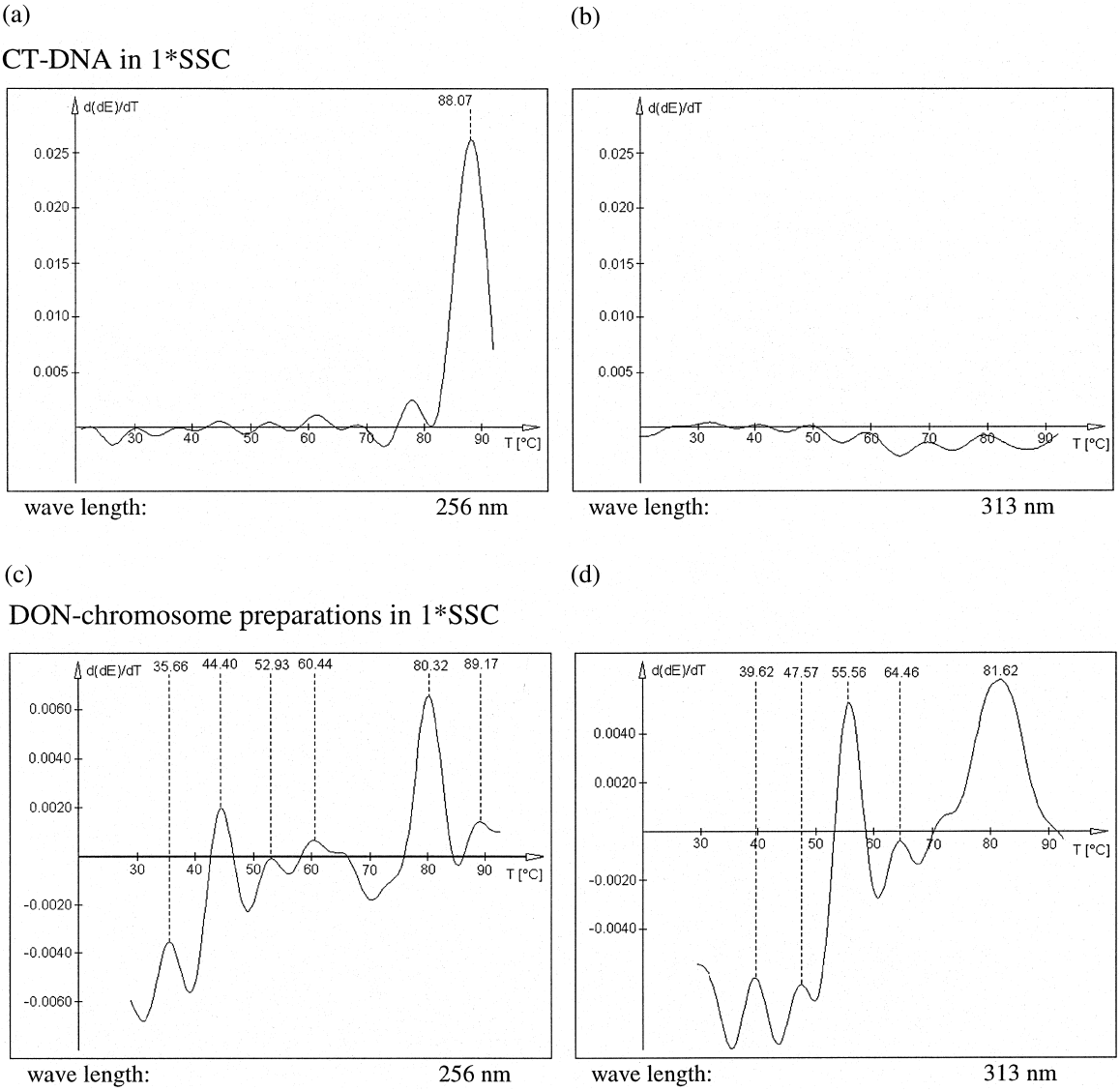


Fig. 3. Melting curves $d(dE)/dT$ vs. T of: (a) Calf thymus-DNA (CT–DNA) in $1 \times$ SSC at 256 nm; and (b) at 313 nm. For metaphase chromosome preparations of cells of the Chinese hamster cell line DON in $1 \times$ SSC, the denaturation profiles are shown in: (c) at 256 nm; and (d) at 313 nm.

vette, so that in case of long term measurement extinction changes due to sedimentation effects may not be excluded.

3. Results

Hyperchromicity measurements were performed for metaphase chromosome preparations and the respective DNA at 256 nm (DNA absorption maximum at neutral pH) and 313 nm (no DNA absorption at neutral pH) excitation maximum. As reference, the same solvent or buffer was taken which was used in the DNA solution or metaphase chromosome preparations. It was expected that under the experimental conditions used, DNA does not absorb light at 313 nm resulting in peaks of the melting curve. In Fig. 2a,b, this is confirmed for DNA of human lymphocytes in 1 × SSC (pH 7). At 256 nm a strong melting peak was found at 88°C which was not detected for 313 nm. In contrast to DNA solutions, conformation changes thermally induced in metaphase chromosome preparations showed a compatible denaturation behaviour at both wavelengths, 256 and 313 nm. As an example, human lymphocyte metaphase chromosome preparations in 1 × SSC were analysed which showed two major peaks at approximately 51°C and 77°C for both wavelengths (Fig. 2c,d) whereas no peak was detected at 88°C.

Such differences in the melting behaviour between DNA solutions and metaphase chromosome preparations were confirmed by other measurements in 1 × SSC comparing CT-DNA with metaphase chromosome preparations of the Chinese hamster cell line DON (Fig. 3). Again a DNA melting point at 88°C was detected for 256 nm which was not found for 313 nm. This melting behaviour was the same for DON-DNA at neutral pH (data not shown). For the metaphase chromosome preparations, two major peaks were found at 44°C and approximately 80°C for 256 nm (Fig. 3c). The latter peak was also visible at 313 nm while the second major peak was at 56°C (Fig. 3d). Taking also smaller peaks into account, conformation changes between 40 and 45°C and between 50 and 55°C were detected for both wavelengths. For 2 × SSC the major peaks were found at 50 to 55°C and 80 to 85°C (data not shown).

In order to show that these peaks are due to higher order structures, DON metaphase chro-

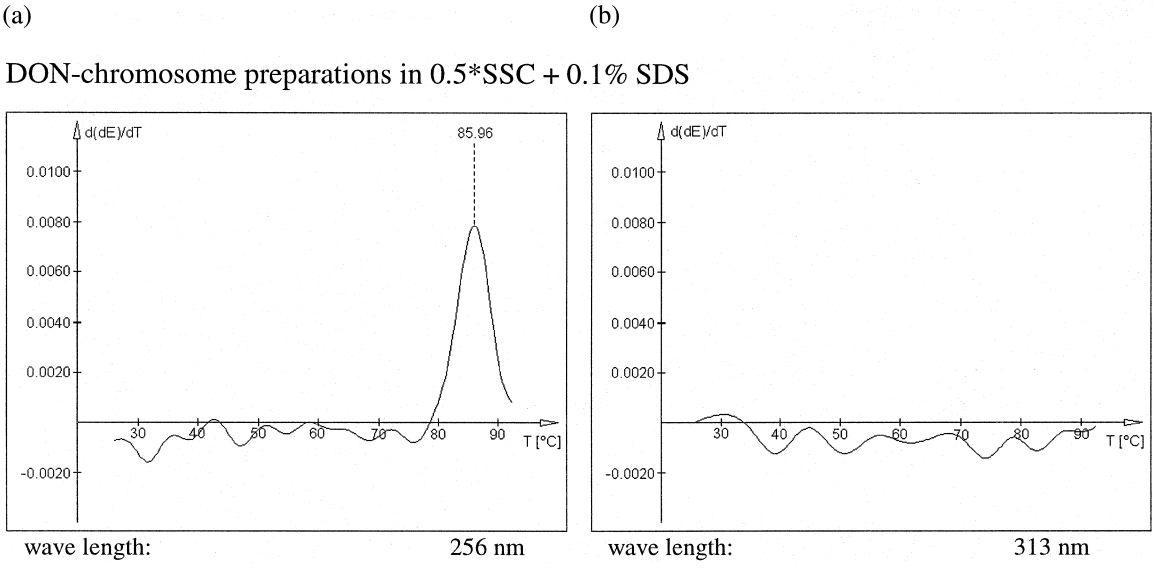


Fig. 4. Melting curves $d(dE)/dT$ vs. T of a DON metaphase chromosome preparation in 0.5 × SSC and 0.1% SDS at: (a) 256 nm; and (b) 313 nm.

Table 1
pH dependent melting point T_M of CT-DNA in 1 × SSC (pH adjusted by acetic acid) obtained from the maximum peak of the melting curves at 256 nm

pH	7.5–4.8	4.2	3.5	3.25	3.1	3.0
T_m	87 ± 1	85 ± 0.5	71 ± 0.5	52 ± 0.5	47 ± 0.5	36 ± 0.5

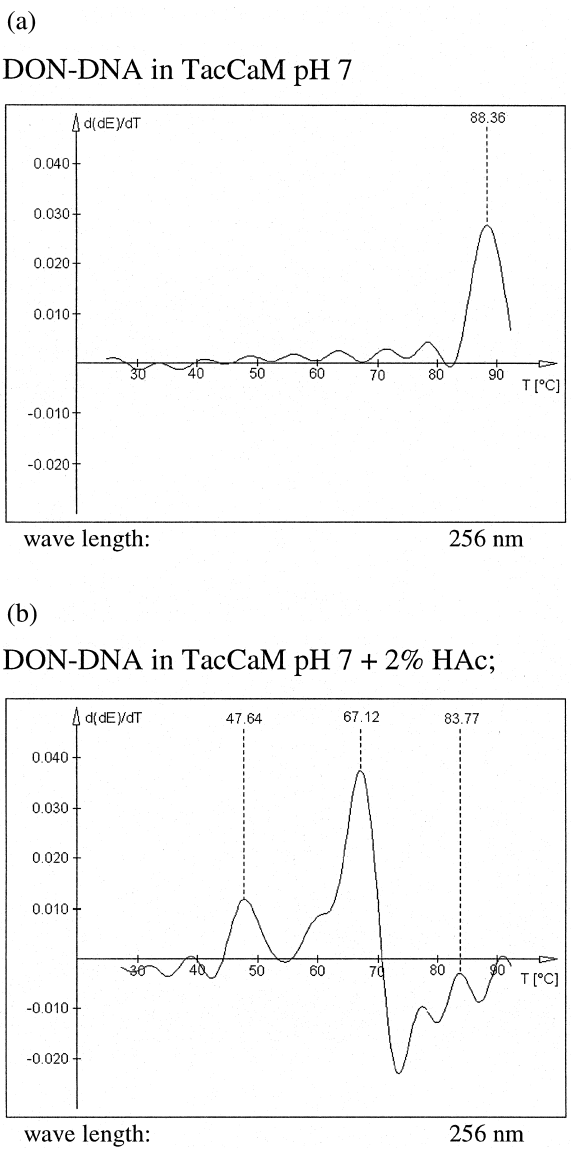


Fig. 5. Melting curves $d(dE)/dT$ vs. T of isolated DON-DNA in chromosome isolation buffer at 256 nm: (a) pH 7; and (b) pH 3.5.

mosome preparations in 0.5 × SSC were exposed to 0.1% SDS (sodium-dodecylsulfate) which is known to dissolve structural proteins. Fig. 4 shows the result which is compatible to the melting curve of pure DNA solutions. Only one peak at 256 nm at 86°C is visible. A corresponding peak at 313 nm is missing.

In another series of measurements the influence of the pH on the reduction of the melting point of DNA was investigated. In Table 1 the melting points (highest temperature with a peak maximum) of CT-DNA in 1 × SSC at different pH values (adjusted by acetic acid) are shown. A similar reduction effect was also found for DON-DNA in the chromosome isolation buffer (Fig. 5). The DNA melting peak shifted from 88°C at pH 7 to approximately 47°C at pH 3.5. For pH 3.5, a second peak was also visible which was ascribed to an extinction change due to light scattering induced by aggregates of single stranded DNA. Therefore, this peak was visible at 313 nm, too. This second peak did not appear in CT-DNA experiments after adding 20% formamide which becomes associated with single stranded DNA so that aggregate formation was avoided (data not shown).

Although a minor peak at approximately 84°C was found, one should be careful in comparing this peak to a melting peak of DNA under neutral pH. This peak may be also due to effects obtained by the cosinus expansion.

These results of DNA solutions were compared to results obtained from suspensions of metaphase chromosome preparations. Human and Chinese hamster cells were used. To eliminate effects due to differences between primary cells and permanent cell lines, the cell lines NC37 (human) and DON (Chinese hamster) were used. At the same

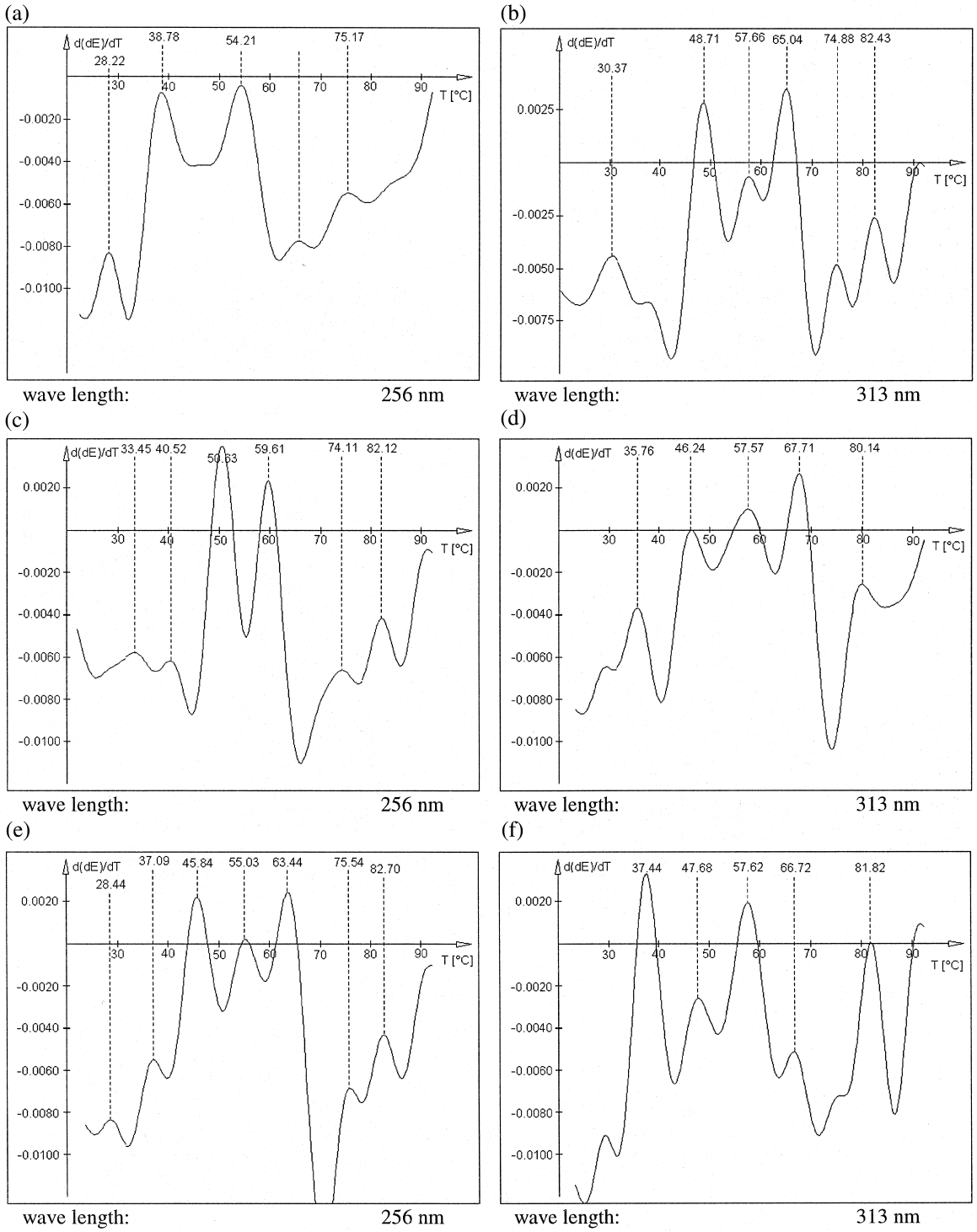
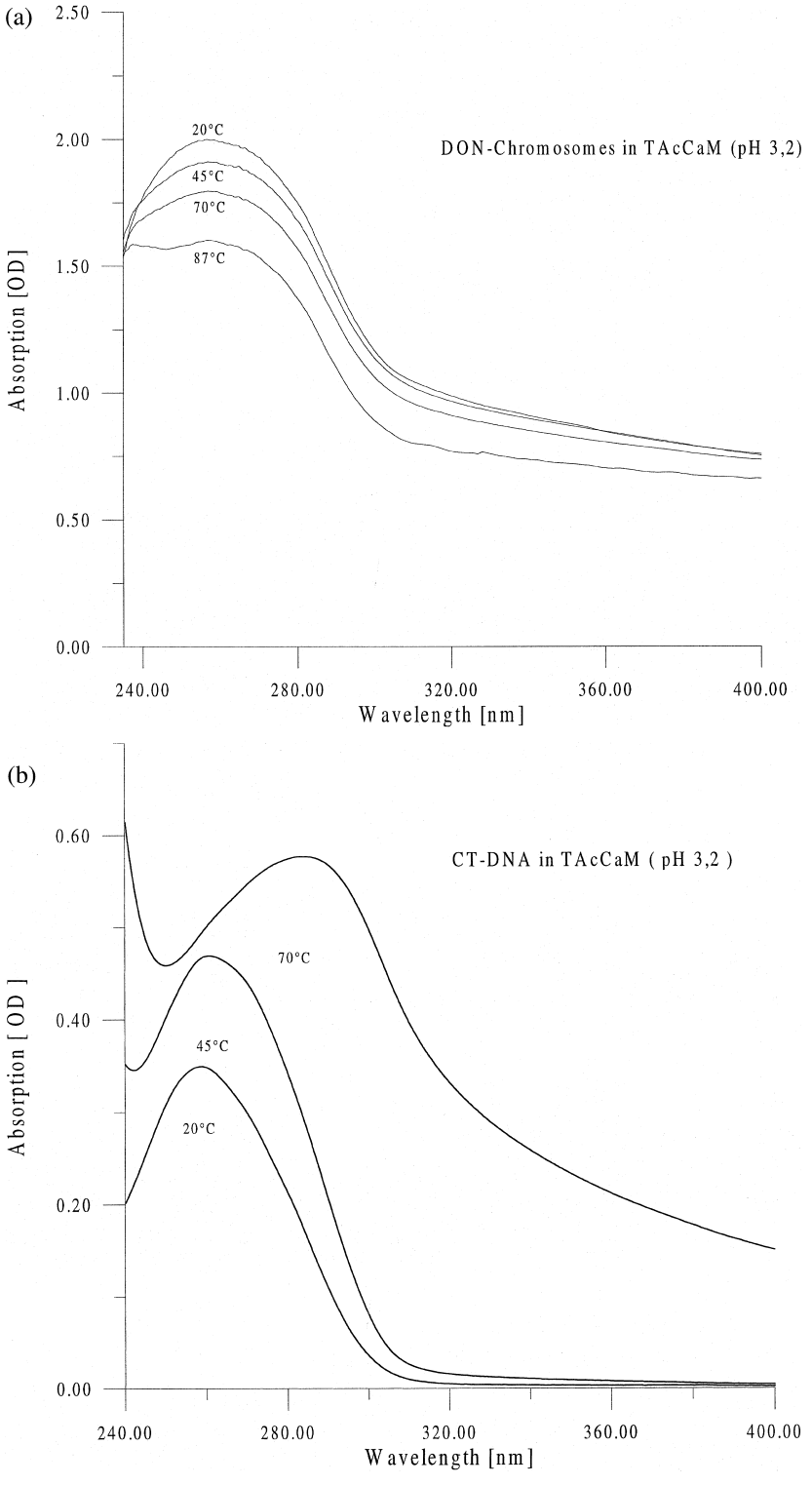


Fig. 6. Typical examples of melting curves $\mathrm{d}(\mathrm{d}E)/\mathrm{d}T$ vs. T of metaphase chromosome preparations of the human cell line NC37 at pH 3.2 prepared in the isolation buffer with hexandiole. (a), (c), (e) three measurements at 256 nm, (b), (d), (f) the corresponding measurements at 313 nm. The examples show the large variability of these types of measurements. However, in contrast to DNA solutions, no significant reduction of the melting point (highest temperature with a peak maximum) was found. In all cases, typically a peak between 75 and 82°C was visible.


(a)
DON-Chromosomes in TAcCaM (pH 3,2)
20°C
45°C
70°C
87°C
Absorption [OD]
2.50
2.00
1.50
1.00
0.50
0.00
240.00
280.00
320.00
360.00
400.00
Wavelength [nm]
(b)
CT-DNA in TAcCaM (pH 3,2)
70°C
45°C
20°C
Absorption [OD]
0.60
0.40
0.20
0.00
240.00
280.00
320.00
360.00
400.00
Wavelength [nm]

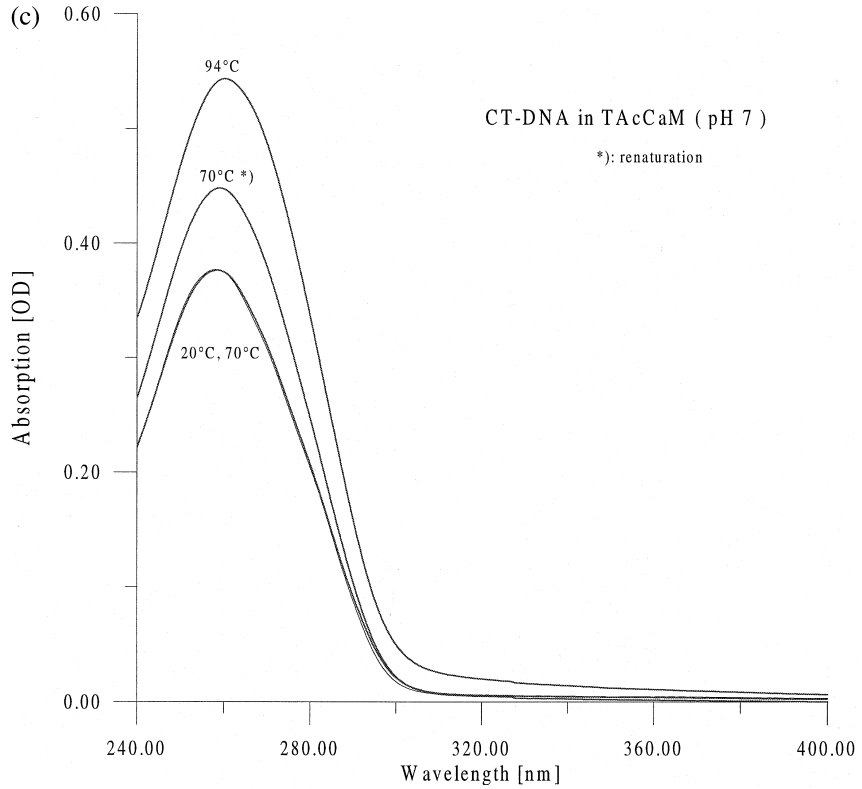


Fig. 7. Absorption spectra (absorption in OD vs. excitation wavelength) of DON-chromosome preparations (a) and CT-DNA solutions (b) in TAcCaM at pH 3.2 measured at different temperatures. In figure (c) the absorption spectra for CT-DNA solutions in TAcCaM at pH 7.0 are shown.

low pH, measurements with and without hexandiole were made. Hexandiole is known to additionally stabilise the structure of metaphase chromosomes and, thus, it was added to test whether this might have an influence on the thermally induced conformation changes registered in the extinction measurements. For DON, no significant difference in the denaturation profiles were found between the isolation buffer with and without hexandiole at low pH (3.2). In all cases a high variability of the peak pattern was found. Independently from the species, at low pH no reduction of the 'melting point' (i.e. the highest temperature with a peak maximum) was found. In all cases, this temperature was between 75 and 82°C (Fig. 6) which was also a characteristic peak region for metaphase chromosome preparations under neutral pH conditions (Fig. 2c,d; Fig. 3c,d).

For the same suspension, the peak patterns at 256 nm and at 313 nm were usually different. However, 96 hyperchromicity measurements for DON chromosome preparations verified those measurements. At pH 3.2 and pH 7.0, compatible peak clusters were observed (J. Rauch, D. Wolf, M. Hausmann, C. Cremer, manuscript submitted).

Although the observation of melting profiles in metaphase chromosome preparations at 313 nm was highly reproducible, its origin from a biophysical point of view was still unclear. For DNA-solutions in $1 \times$ SSC or TAcCaM at pH 7.0, no effect was detected at 313 nm. At pH 3.2, typically only that peak which was found to be probably caused by extinction changes due to light scattering of aggregates was also visible at 313 nm. To achieve a better physical basis for the results obtained for metaphase chromosome preparations, absorption spectra between 230 nm and 400 nm were measured at different temperatures.

In Fig. 7a the results for DON-chromosome preparations in TAcCaM at pH 3.2 are shown for four temperature values (20, 45, 70, and 87°C). All curves were very similar in their shape and showed a broad maximum centred approximately 260 nm, with decreasing values at higher wavelengths. With increasing temperature, the absorption decreased. However, for the complete wavelength range measured the absorption remained over a certain level and did not reach zero.

The absorption spectra of CT-DNA in TAcCAM at pH 3.2 were completely different (Fig. 7b). Below 45°C they showed a peak with a maximum at approximately 260 nm and negligible absorption at 310–320 nm. Above 50°C the peak shifted to higher values. For 70°C the maximum was at approximately 290 nm with a broad absorption background over the complete wavelength range measured.

This was completely different to measurements of CT-DNA in TAcCaM at pH 7.0 (Fig. 7c). For all temperatures between 20°C and 94°C the peak position was at approximately 260 nm. Between 70°C and 94°C the absorption peak increased due to DNA melting. For wavelengths above 320 nm the absorption was negligibly small.

4. Discussion

Hyperchromicity measurements are well established to analyse DNA melting. Usually the temperature dependent extinction at approximately 260 nm of DNA solutions is measured. To compare such data to conformation changes of higher order structures such as chromosomes it has to be considered that these latter are particle suspensions, since typical dimensions of mammalian metaphase chromosomes are in the range of 1–10 μm. Due to sedimentation effects the extinction may change during long term measurements with standard photometers having a horizontal optical axis. To measure the temperature depending extinction both of suspensions and solutions, with the same device, a high-resolution, computer controlled photometer with a vertical optical axis was designed and constructed [19,21,22].

A computer program calculating the first derivative of an extinction function was written [20] to obtain a sample specific peak pattern. This peak pattern obtained was expected to be influenced not only by changes in absorption but also by changes in light scattering. Since both processes are connected with DNA/chromosome conformation (secondary and tertiary structure), thermally induced conformation changes can be represented by a typical peak at a certain temperature.

In order to obtain a continuous extinction function, the expansion by a cosinus series was used. Thus, a coherent denaturation profile was obtained over the complete temperature range of interest. However, two effects of the cosinus expansion have to be considered:

1. The boundary values have to be appropriately adapted to gradient zero. This may generate an additional peak at the boundary which has to be excluded from consideration.
2. The cosinus terms generate a harmonic background which becomes visible in cases where $\mathrm{d}(\mathrm{d}E)/\mathrm{d}T$ is close to zero, e.g. if sample and reference are identical (see e.g. Fig. 2b, and Fig. 3b). This background has to be considered as the optical resolution in order not to overestimate background fluctuations as denaturation peaks [21].

The suitability of the device was tested by comparing DNA solutions with metaphase chromosome preparations of two species (human and Chinese hamster). At neutral pH no species depending difference in DNA melting of commercially available calf thymus (CT), DON (Chinese hamster) and human (lymphocyte) DNA was found. Some tens of measurements were made confirming the high reproducibility with which the system was running. This was helpful to define a DNA standard for test measurements, for example, CT-DNA. As expected, decreasing the pH down to 3.0, the melting temperature was reduced.

In contrast to DNA where typically one denaturation peak was found, the more complex structures of metaphase chromosome preparations led

to more complex denaturation profiles. Under the same buffer conditions, the temperature values of conformation changes of metaphase chromosome preparations, did not directly correlate with the melting temperatures of the DNA solutions. However, independently from the cell type (human, Chinese hamster) two major regions of conformation changes were identified: one between 75 and 82°C the other between 40 and 55°C.

With the reduction of the pH to 3.2, conformation changes in metaphase chromosome preparations were not restricted to lower temperatures only as it was typically found for pure DNA. Moreover, independently from the species the peak with the highest temperature was again found in the range between 75 and 82°C. All peaks showed a large variability in their number and position. The melting curves of metaphase chromosome preparations at 256 and 313 nm were compatible concerning this pattern. The absorption spectra measured at pH 3.2 showed a considerable absorption background over the complete wavelength range measured. This background can be interpreted as a signal reduction induced by light scattering of particles. The same behaviour was found for DNA at pH 3.2 over 50°C when aggregates were formed. However, in contrast to DNA, the absorption curves of metaphase chromosome preparations did not change their principle shape at higher temperatures, but reduced their absolute values. Thus, the peaks in the melting profiles of metaphase chromosome preparations may be interpreted as denaturing effects due to conformation changes and not only as absorption effects by increased light scattering.

To draw further conclusions from such peak patterns concerning the biophysical mechanisms of chromosome denaturation (changes in the secondary and tertiary structure) extended series of measurements under defined buffer conditions with a statistical evaluation are required. So far many details in these measurements and their possible correlations to conformation changes are not understood. Such evaluations will be the task of further experiments. For these experiments also a standardised cell type appears to be useful, for example, a Chinese hamster or human cell line.

Here, we showed the suitability of the device described above, to directly compare the melting behaviour of DNA solutions with the denaturation of metaphase chromosome preparations in suspension. The data so far obtained indicate that metaphase chromosome preparations show a completely different denaturation behaviour compared to pure mammalian DNA, supporting the idea of a high structural complexity of chromosomes [14]. Under the preparation conditions used, the typical denaturation behaviour was independent for both types of sample; this is compatible with the view that the structural complexity of chromosomes is a universal phenomenon.

It is anticipated that comparative experiments of DNA and metaphase chromosome preparations may be interesting to analyse the denaturation and especially renaturation behaviour of DNA probes and chromosomal targets for non-enzymatic (fluorescence) in situ hybridisation (FISH) [23]. Up to now, the photometrical setup described is only useful to study denaturing effects since no active cooling is available. However, recent experiments of probe to target renaturation and probe to probe reassociation obtained by quantitative microscopy after fluorescence in situ hybridisation indicated that the chromosomal target DNA was still in a denatured condition even 20 h following heating at 85 or 92°C and therefore still accessible to labelling probes [24]. These results make the full reversibility of the measured denaturation effects shown here questionable. A lack of reversibility again is in accordance with a highly complex native structure of chromosomes. Effects of routinely used denaturing chemical agents, e.g. formamide, on probe and target together with heating may be analysed in comparison to thermal treatment only; this latter has become an important procedure in Fast-FISH [25,26]. Such protocols have the potential also to label chromosomal targets under physiological temperature conditions [27]. For the development of such protocols not only the renaturation behaviour but also the denaturation behaviour of chromosome targets are of high inter-

est. The device and basic experiments here offer the possibility to systematic studies to optimise FISH protocols (J. Rauch, D. Wolf, M. Hausmann, C. Cremer, manuscript submitted), especially under more physiological conditions compared to the commonly used ones.

Acknowledgements

The authors gratefully acknowledge the contributions of Dr U. Bettag, T. Velten and Dr S Heiland in the development of the photometer and the contribution of D. Adam in its characterisation. The financial support of the Deutsche Forschungsgemeinschaft is also gratefully acknowledged. The authors are furthermore indebted to H. Ludwig, Physikalisch-Chemisches Institut, Heidelberg, for the access to the PERKIN ELMER Lambda 14 Spectrometer.

References

[1] A. Akhrem, V. Andrianov, A. Vlasov, N. Korolev, N. Kuznetsov, Mol. Biol. (Mosk) 19 (1985) 623.
[2] A.T. Ansevin, D.L. Vizard, B.W. Brown, J. McConathy, Biopolymers 15 (1976) 153.
[3] Y.P. Blagoi, V. Sorokin, V. Valeyev, S. Knomenko, G. Gladchenko, Biopolymers 17 (1978) 1103.
[4] J. Marmur, P.O.P. Ts'o, Biochim. Biophys. Acta 51 (1961) 32.
[5] C. Schildkraut, Biopolymers 3 (1965) 195.
[6] J. Marmur, P. Doty, Nature 183 (1959) 1427.
[7] J. Marmur, P. Doty, J. Mol. Biol. 5 (1962) 109.
[8] A.T. Ansevin, Methods in Cell Biology XVIII, Academic Press, New York, 1978, p. 397.
[9] H.J. Li, J. Bonner, Biochemistry 10 (1971) 1461.
[10] H.J. Li, C. Chang, M. Weiskopf, Biochemistry 12 (1973) 1763.
[11] H.J. Li, in: H.J. Li, R. Eckhard (Eds.), Chromatin and Chromosome Structure, Academic Press, New York, 1976, p. 37.
[12] H.J. Li, Methods in Cell Biology XVIII, Academic Press, New York, 1978, p. 385.
[13] T.Y. Shih, R.S. Lake, Biochemistry 11 (1972) 4811.
[14] T. Cremer, A. Kurz, R. Zirbel et al., Cold Spring Harbor Symp. Quant. Biol. 58 (1993) 777.
[15] R.K.A. Schaller, R.B. Spiess, F.F. Bier, U. Bettag, C. Cremer, Biophys. Chem. 38 (1990) 59.
[16] D.T. Arakaki, R.S. Sparkes, Cytogenetics 2 (1963) 57.
[17] P.S. Moorhead, P.C. Nowel, W.J. Mellham, B.M. Battips, D.A. Hungerford, Exp. Cell Res. 20 (1960) 613.
[18] M. Stöhr, K.J. Hutter, M. Frank, K. Goerttler, Histochemistry 74 (1982) 57.
[19] U. Bettag, Doctoral thesis, University of Heidelberg, 1989.
[20] T. Velten, Diploma thesis, University of Heidelberg, 1989.
[21] D. Adam, Diploma thesis, University of Heidelberg, 1993.
[22] S. Heiland, Diploma thesis, University of Heidelberg, 1989.
[23] P. Lichter, T. Cremer, in: D.E. Rooney, B.H. Czepulkowski (Eds.), Human Genetics — A Practical Approach, IRL Press, Oxford, 1992, p. 157.
[24] D. Wolf, Doctoral thesis, University of Heidelberg, 1998.
[25] M. Durm, F.M. Haar, M. Hausmann, H. Ludwig, C. Cremer, Z. Naturforsch. C 51 (1996) 253.
[26] F.M. Haar, M. Durm, M. Hausmann, H. Ludwig, C. Cremer, J. Biochem. Biophys. Meth. 33 (1996) 43.
[27] M. Durm, F.M. Haar, M. Hausmann, H. Ludwig, C. Cremer, Z. Naturforsch. C 52 (1997) 82.