The Influence of Formamide on Thermal Denaturation Profiles of DNA and Metaphase Chromosomes in Suspension

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Systematic photometric studies are presented to analyze the thermal denaturation behaviour with and without formamide of metaphase chromosome suspensions in comparison to DNA solutions. Temperature dependent hyperchromicity measurements at 256 nm and 313 nm were performed using an appropriately designed computer-controlled photometer device. Due to an upright optical axis, this allowed absorbance measurements with negligible sedimentation effects not only for solutions of pure DNA, but also for particle suspensions of isolated metaphase chromosomes. This device has a temperature resolution of ± 0.5 °C and an optical sensitivity of 10^{-3} to 10^{-4} optical density. For calf thymus DNA the reduction of the melting point with the increase of formamide in the solution was measured at pH 7.0 and pH 3.2. The good correlation of the theoretical approximation to experimental data indicated the suitability of the apparatus to quantitatively describe DNA conformation changes induced by thermal denaturation. For metaphase chromosome preparations of Chinese hamster culture cells, absorbance changes were measured between 20 °C and 95 °C with a temperature gradient of 1 °C/min. These measurements were performed at pH 7.0 and at pH 3.2. The denaturation profiles (= first derivative of the absorbance curve) resulted in a highly variable peak pattern at 256 nm and 313 nm indicating complex conformation changes. A statistical evaluation of the temperature values of the peak maxima resulted in temperature ranges typical for chromosomal conformation changes during thermal treatment. Especially the range of highest temperature values was independent from pH modifications. For pH 3.2 the influence of formamide on the denaturation behaviour of metaphase chromosome preparations was analyzed. In contrast to pure DNA solutions, a reduction of the "melting point" (i.e. the maximum temperature at which a conformation change takes place) was not found. However, the denaturation behaviour depended on the duration of formamide treatment before the measurement.

Introduction

Thermally induced conformation changes of DNA, especially strand separation (also called "DNA denaturation" or "DNA melting") depend on the in situ chemical conditions of the DNA for instance the presence of positive ions (Akhrem *et al.,* 1985; Ansevin *et al.,* 1976; Blagoi *et al.,* 1978; Marmur and Ts'o, 1961; Schildkraut, 1965). DNA melting is usually measured photometrically by the temperature dependent absorbance at 260 nm (Marmur and Doty, 1959; Marmur and Doty, 1962; Ansevin, 1978). The transition probability of the π-electrons of the DNA bases corresponding to absorbency is about 30% higher for single stranded DNA than for double stranded. Thus, DNA melting shows a typical step-like hyperchromicity (Ansevin *et al.,* 1976; Ansevin, 1978). The melting-point (T_M) is defined as the temperature at which 50% double-stranded and 50% single-stranded DNA exist. This is the point of inflection in absorbance curves (absorbance change dE vs. temperature T) and corresponds to a peak maximum at T_M in the first derivative of the hyperchromicity curve. In the following this peak profile is called melting curve or denaturation profile, respectively.

Formamide is often used as a denaturating chemical agent which influences the DNA melting behaviour. This means that an increase in the for-

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mamide concentration decreases the T_M. For instance, it has been reported that for sodium chloride concentrations ranging from 0.035 M to 0.88 M, an increase from 0% to 50% formamide lowers the T_M by 0.6 °C per percent formamide (Hutton, 1977).

Based on calculations for DNA (Schidkraut, 1965; Meinkoth and Wahl, 1984), high amounts of formamide are not only used to reduce the melting point of pure DNA solutions but also to influence the denaturation behaviour of higher order DNA-protein complexes, e.g., chromosomes or chromatin, in such a way that due to temperature reduction the native structure of such complexes may be better maintained. This, however, means that under the certain experimental conditions chromosomal DNA with its complete protein backbone should show a similar melting behaviour like pure DNA in solution. Recent investigations on new fluorescence in situ hybridization (FISH) protocols, in which in contrast to other established protocols (Lichter and Cremer, 1992; Carter, 1996) formamide or other denaturing chemical agents were omitted (Durm *et al.*, 1996; Durm *et al.*, 1998; Haar *et al.*, 1996), indicated that pure DNA and chromosomes are more different concerning their denaturation behaviour than expected.

Therefore it appeared to be interesting to develop a photometric device to investigate DNA denaturation *and* chromosome denaturation according to same protocol of absorbance measurements (Wolf *et al.*, 1999), since hyperchromicity at 260 nm is a long established parameter to analyze DNA melting. In contrast to pure DNA, metaphase chromosome preparations are suspensions of particles subjected to sedimentation during long term (i.e. hours) measurements. To overcome spurious "hyperchromicity effects" induced by particle sedimentation, an appropriately designed computer controlled photometer with a vertical optical axis was built (Wolf *et al.*, 1999; Bettag, 1989; Heiland, 1989; Velten, 1989). Absorbance measurements at about 260 nm and about 320 nm were performed to obtain denaturation profiles of metaphase chromosome preparations of Chinese hamster culture cells (DON) under different chemical conditions in comparison to calf thymus (CT) -DNA. In contrast to DNA obtained from cells of the Chinese hamster cell line DON (DON-DNA), CT-DNA was commercially available. In former experiments, it has been proved to be compatible with Chinese hamster DNA according to its base composition and denaturation behaviour (Wolf *et al.*, 1999; Schaller *et al.*, 1990).

Materials and Methods

Metaphase chromosome preparations

Cultured cells of the Chinese hamster cell line DON were used in order to have highly reproducible cell conditions for all experiments. Recent results (Wolf *et al.*, 1999) indicated that metaphase chromosome suspensions of this cell line showed a denaturation behaviour compatible to human metaphase chromosome suspensions concerning the photometric studies presented here. Therefore the DON cell line may be used as a model system.

Mitotic cells were synchronized by a Colcemid block of 4 h using 0.25 µg Colcemid (demecolchine, N-deacetyl-N-methylcolchicine) in 1 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 (trishydroxymethylaminomethane hydrochloride), 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 "TAcCaM" isolation buffer (25 mM **T**ris-**Ac**etic acid, 5 mM **Ca**Cl_2, 5 mM **Mg**Cl_2, pH 3.2) was added (Wolf *et al.*, 1999; Schaller *et al.*, 1990). In this isolation buffer, the mitotic cells (presumed to be mostly in metaphase) were syringed to disrupt the cell membrane and to get a suspension with dispersed chromosomes. For the experiments at pH 7.0, the metaphase chromosome suspension was again centrifuged (150×*g*, 10 min) and the TAcCaM isolation buffer of pH 3.2 was replaced by the same buffer adjusted to pH 7.0 by a lower amount of acetic acid.

200–300 µl of the original metaphase chromosome suspensions were diluted to a final concentration of approximately 20 µg chromosomes/ml. For the experiments with formamide, the relative amount was added to the total volume of the suspension. Before the absorbance measurements, these suspensions were incubated for different times.

The sample suspension and the reference solution (pure buffer solution) were degased for half an hour to avoid air bubbles during the measurement. For each measurement, 150 µl sample suspension and 150 µl reference solution were used in the photometer cuvettes.

DNA solutions

Commercially available calf thymus DNA (CT-DNA) (type I, "highly polymerized", Sigma GmbH, Deisenhofen, Germany) was used for comparison with the DON Chinese hamster metaphase chromosome preparations. Due to its base composition this DNA is according to hyperchromicity measurements compatible to Chinese hamster DNA (Wolf *et al.,* 1999; Schaller *et al.*, 1990). Using commercially available DNA had the advantage of a highly reproducable quality.

For hyperchromicity measurements the DNA was solved in 1× SSC (150 mM NaCl, 15 mM Tri-Na-Citrate) or TAcCaM at a final concentration of about 20 µg DNA/ml solution. Formamide was added in relative amounts. After degasing 150 µl DNA solution and 150 µl buffer without DNA (reference) were used in the photometer cuvettes.

The CT-DNA used had an average strand length of n=27 kb and an average guanine cytosine ratio of 42%. Thus the formamide depending melting point was approximated to T_M[°C] = 86.52–0.61 (% formamide) according to the formula

$$T_M\,[°C] = 81.5 + 16.6 \log \bar{M} + 0.41\,(\%G + \%C) - 500/n - 0.61\,(\%\ \text{formamide})$$

with: $\bar{M}$ = concentration of positive ions [mol/l] (3×310^{-4} M ≤ $\bar{M}$ ≤ 0.5 M); %G and %C = the percentage of guanine or cytosine bases, respectively (25% ≤ %G, %C ≤ 75%); n = strand length (n > 50) (Schildkraut, 1965; Meinkoth and Wahl, 1984).

Vertical path-way photometer

To investigate the thermally induced absorbance of solutions and particle suspensions in cuvettes, 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 (Bettag, 1989) and described in detail in (Wolf *et al.,* 1999). This vertical optical pathway has the advantage that stirring in the cuvette is not necessary and very small amounts of chromosome suspensions (150 µl) can be used for the measurements. The entire device consists of three functional subunits:

1) the optical system with a specially designed, exactly heatable cuvette for probe and reference; excitation takes place at 256 nm or 313 nm using appropriate bandpass filters;
2) the electronic control unit for signal detection, temperature registration, and the heating;
3) the data processing unit for digitization and computing of temperature and absorbance data and online control of the system.

Data processing

For data processing and graphical representation the original data are rescaled into absolute temperature values T[°C] and the first derivative of absorbance curves $d(dE)/dT$ [OD/°C] (OD: = optical density) was plotted. Here, dE is derived from the Lambert-Beer law. The absorbance is given as the logarithm $\log_e$ of the ratio of the light intensity passing the reference $I_{ref}(T)$ and that passing through the sample $I_s(T)$. dE is defined as the difference between the absorbance at the actual temperature T and the initial temperature T_o on basis of $\log_{10}$:

$$dE = \log_{10} e \cdot (\log_e E(T) - \log_e E(T_0))$$

$$dE = \log_{10} e \cdot \left(\log_e \frac{I_{ref}(T)}{I_s(T)} - \log_e \frac{I_{ref}(T_o)}{I_s(T_o)} \right).$$

In order to obtain a continous profile over the entire temperature range analyzed, an expansion by a linear combination of cosinus functions was applied on the measured values (for details see: Wolf *et al.,* 1999; Velten, 1989). Note: dE as well as $d(dE)/dT$ may also become negative. For the analysis of denaturation profiles, however, only the positions of peaks T_M are relevant. In this content a peak is a local maximum of $d(dE)/dT$ with a difference to the neighbouring local minima which is higher than the apparative resolution.

Apparative resolution

The effective temperature resolution of the photometer was ±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 *d(dE)/dT* versus *T*. Using H_2O as sample and reference, the optical resolution was measured by the optical density and estimated to be 1.1×10^{-3} for 256 nm excitation and 3.0×10^{-4} for 313 nm (Adam, 1993).

Results

CT-DNA

Denaturation profiles of pure CT-DNA dissolved in 1× SSC and TAcCaM (pH 7.0) were measured as a standard to test the reliability of the apparative device and of the automatic data processing. Fig. 1 shows typical examples. The denaturation peak at 88 °C (1× SSC) or 86 °C (TAcCaM, pH 7.0) is only visible at 256 nm excitation since pure DNA does not absorbe at 313 nm under these chemical conditions. Due to the high number of conformation changes expected in the denaturation profiles of chromosomes in suspension (= high number of denaturation peaks, see below), it appeared useful to smooth the denaturation profiles slightly by expanding with a linear combination of cosinus functions up to not more than the 20th order. This, however, suppressed the fine structure of the DNA melting curves usually obtained by satellite sequences. Because compatible structures were also not detectable in the denaturation profiles of chromosomes in suspension, this lack appears to be tolerable.

For increasing percentages of formamide (0%–80%), the melting point T_M was determined by the peak position in the denaturation profiles for both neutral solvents (Figs. 2a, 2c). A linear decrease of the melting point was obtained. These data were compared to the theoretical approximation (T_M[°C] = 86.52–0.61 (% formamide)) (Fig. 2b). Up to 60% formamide the theoretical approximation was in good correlation to the 1× SSC data. However, we did not succeed to verify that the approximation is still valuable up to 75% formamide. The melting temperature in TAcCaM pH 7.0 was lower than in 1× SSC up to 60% formamide. For 70% and 80% formamide it was

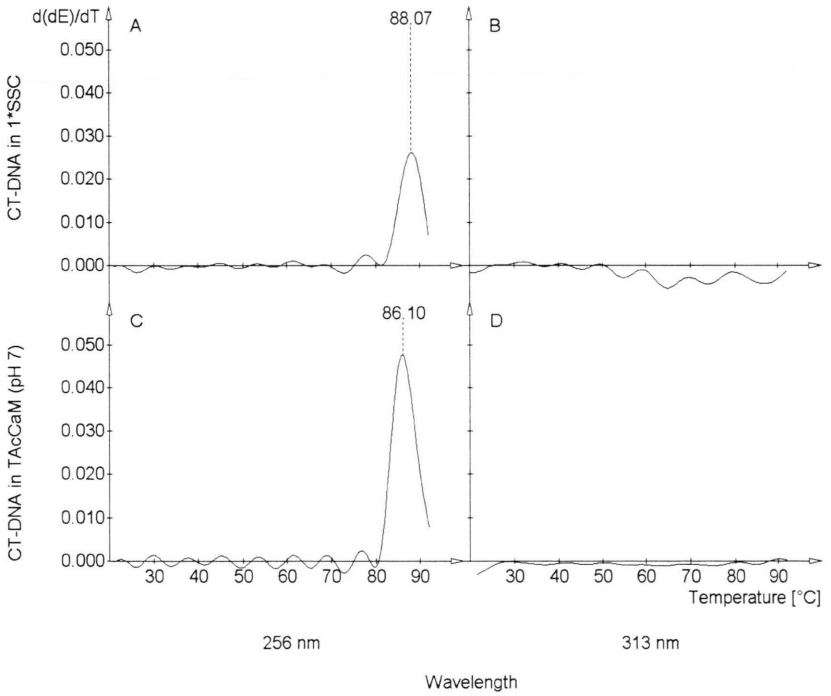

Fig. 1. Denaturation profiles *d(dE)/dT* versus *T* for calf thymus DNA (CT-DNA) in 1× SSC (a,b) and TAcCaM at pH 7 (c,d). a), c) 256 nm excitation; the peak position represents the melting point at 88 °C or 86 °C, respectively; b), d) 313 nm excitation; (no DNA absorbance visible). Note: small fluctuations in the curves are due to the cosinus expansion.

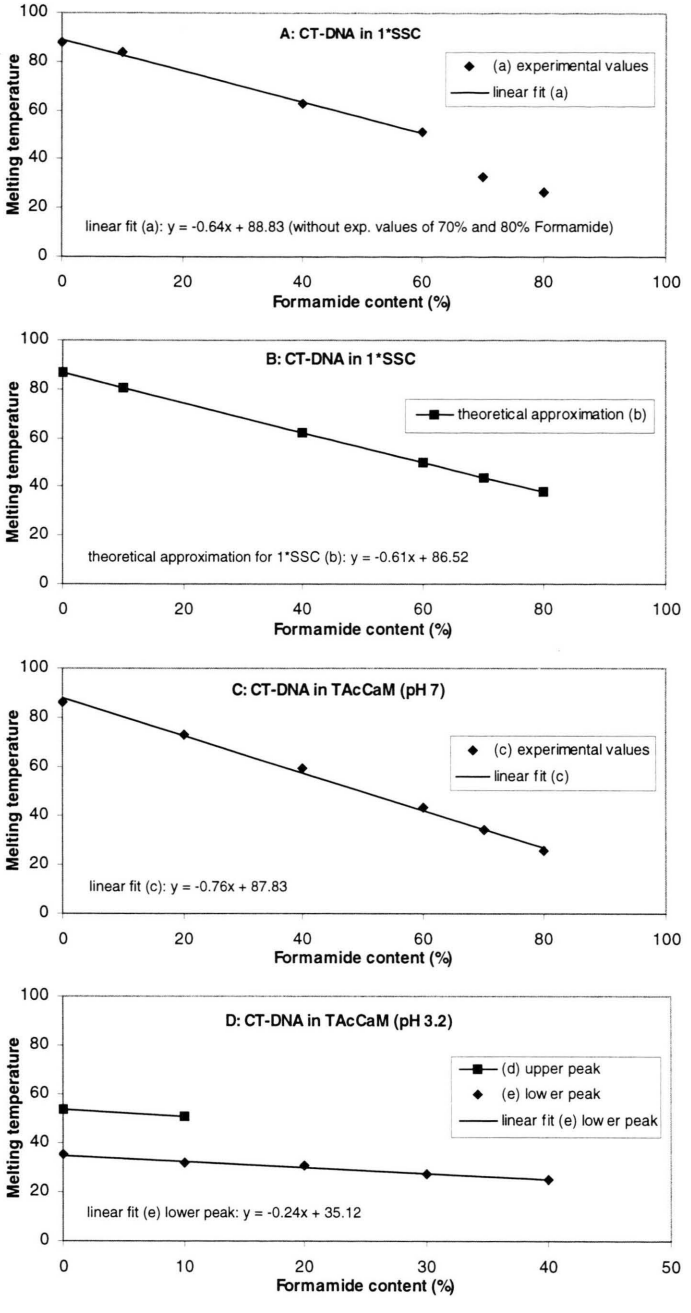

Fig. 2. Melting point T_M versus relative formamide content for calf thymus DNA (CT-DNA): a) experimental values for 1× SSC; b) theoretical approximation for 1× SSC; c) experimental values for TAcCaM pH 7 and d) TAcCaM pH 3.2. The data were obtained from the peak positions in the denaturation profiles at 256 nm.

higher which may be due to the divalent ions Mg^{2+} and Ca^{2+} in TAcCaM.

Since several chromosome measurements were performed in the isolation buffer TAcCaM at pH 3.2 to maintain the particle morphology of the suspended chromosomes (Hausmann *et al.*, 1993), the influence of formamide on the CT-DNA melting point was also measured for TAcCaM pH 3.2 (Fig. 2d). In contrast to TAcCaM pH 7.0, CT-DNA showed two peaks for 256 nm and one peak at 313 nm with a temperature value which was corresponding to one 256 nm

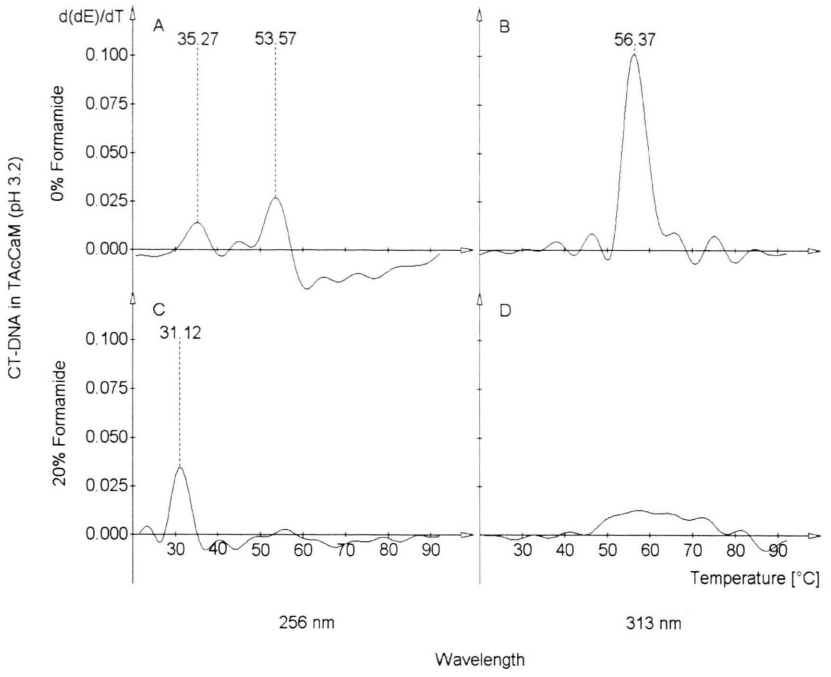

Fig. 3. Denaturation profiles *d(dE)/dT* versus *T* for calf thymus DNA (CT-DNA) in TAcCaM pH 3.2 without formamide (a) 256 nm excitation; b) 313 nm excitation), and with 20% formamide (c) 256 nm excitation; d) 313 nm excitation).

peak (Fig. 3a, b). With increasing the formamide content to more than 20%, this peak disappeared for both excitation wavelengths (Fig. 3c, d). Since absorbance changes at 313 nm cannot be explained by DNA alone, this peak can be ascribed to light scattering effects. CT-DNA in TAcCaM pH 3.2 showed aggregation effects which were responsible for light scattering at higher temperatures. This interpretation was supported by the spectral absorption of DNA at different temperatures (see: Wolf *et al.*, 1999).

With an increase of the percentage of formamide, the formation of these DNA aggregates was suppressed and only the true denaturation peak of the melting profile remained at 256 nm. As for the TAcCaM pH 7.0 buffer, the melting point decreased linearly with increasing percentage of formamide (Figs. 2c, 2d). However, the linear regression revealed a lower gradient which was not compatible with the T_M-formula (Fig. 2b).

Metaphase chromosome suspensions

In contrast to DNA solutions, suspensions from metaphase chromosome preparations showed a completely different denaturation behaviour in photometric measurements. Typically *d(dE)/dT* was about one order of magnitude lower than for DNA. It can be estimated that the reduction in the amount of DNA was about a factor of two, so that the differences in *d(dE)/dT* were assumed to be due to DNA protein interactions and higher order DNA structures. For 256 nm and 313 nm, metaphase chromosome preparations showed a series of peaks in the denaturation profile covering the complete temperature range of the measurements. The peaks detected at 256 nm were in good correlation to those detected at 313 nm for aliquots of the same preparation (Fig. 4). Spectral absorption measurements (see Wolf *et al.*, 1999) at certain constant temperatures showed that in contrast to CT-DNA in TAcCaM pH 3.2, these peaks were not correlated to aggregates in the suspension.

Although the preparative conditions (cell line, isolation protocol etc.) were identical for all mea-

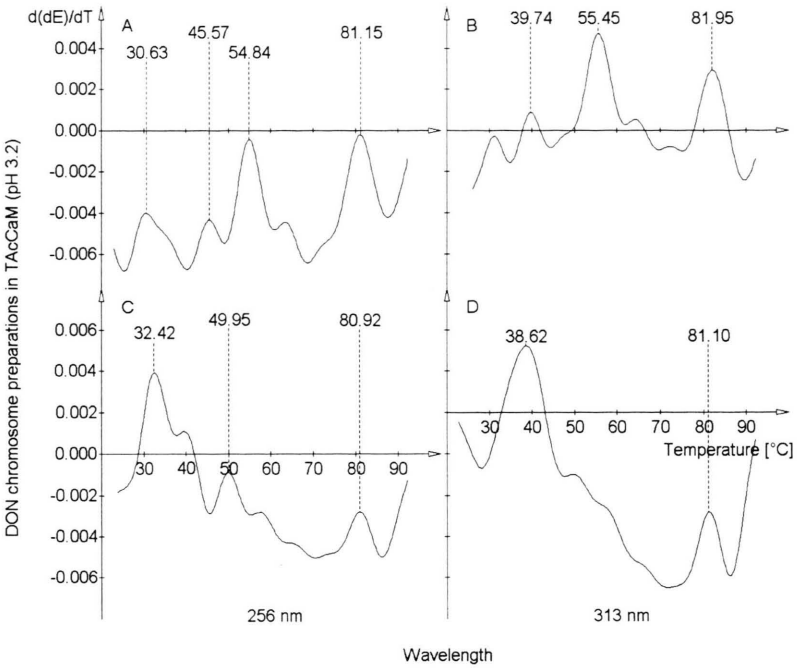

Fig. 4. Typical examples of the denaturation profiles *d(dE)/dT* versus *T* for metaphase chromosome suspensions of the Chinese hamster cell line DON. Shown are two measurements at 256 nm excitation (a,c) and two measurements at 313 nm excitation (b,d) of aliquots of the same two suspensions. Although the preparation conditions were identical, a high variability in the peak pattern was found. The major peaks obtained at 256 nm showed a good correlation to those obtained at 313 nm for each two aliquots of the same suspension.

surements with the same pH, the peak pattern showed a high variability especially in the temperature range below 70 °C. A typical peak, however, was found at about 80 °C independently of pH (pH 7.0 or pH 3.2). The temperature values of the peaks of 80 measurements at pH 3.2 and 16 measurements at pH 7.0 were summarized in weightened frequency histograms (Fig. 5). The pattern of these histograms were similar for pH 7.0 and pH 3.2. In both cases, broader groups of peaks were visible below 70 °C. Typically no peak was found between 71 °C and 77 °C for both pH values. In the temperature range at about 80 °C an increased frequency of peaks was found. For pH 7.0 this was in good correlation to the melting temperature of pure DNA (see also: Wolf *et al.*, 1999) without formamide (compare Figs. 1, 2c). However, such a correlation was not found at pH 3.2 (compare Figs. 3a, 2d).

To investigate a possible effect of formamide on the complex conformation changes of metaphase chromosomes in suspension during thermal treatment, a series of denaturation profiles using 20% formamide in the buffer TAcCaM pH 3.2 was registered. This value was choosen because it was expected from the DNA measurements that aggregrates may be still suppressed. On the other hand higher values of formamide led to an increase in buffer absorption so that many peak heights would become similar to apparative resolution.

Denaturation profiles were registered at 256 nm and 313 nm for formamide treatment of 10 min, 2 hrs and 1 day before the measurement. Again the corresponding measurements at 256 nm and 313 nm showed a compatible peak pattern. The temperature range 68 °C – 90 °C is the usually applied range of target denaturation in established *in situ* hybridization protocols. In this range an accumulation of absorbance changes at about 80 °C was found typical for metaphase chromosome suspensions. Thus, we defined this accumulated peak frequency with the temperature of "complete denaturation". Table I gives the mean values of "complete denaturation" without and with for-

Table I. Range of "complete denaturation" (68 °C – 90 °C) obtained from denaturation profiles of metaphase chromosome preparations of the Chinese hamster cell line DON.

Conditions of formamide treatment	Mean temperature of denaturation peak 1 [°C]	Mean temperature of denaturation peak 2 [°C]
Without formamide treatment	80.9 ± 1.3	– –
Formamide treatment: 10 min	79.7 ± 0.8	– –
Formamide treatment: 2 hours	74.3 ± 1.7	82.6 ± 1.6
Formamide treatment: 1 day	71.4 ± 1.0	82.3 ± 1.6

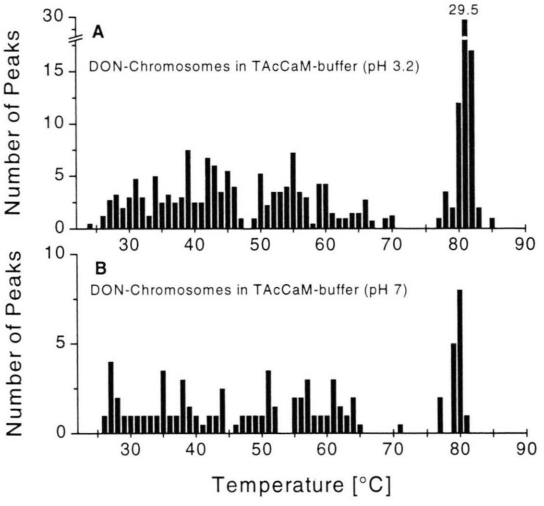

Fig. 5. Frequency histograms of peaks at a given temperature obtained from denaturation profiles. *d(dE)/dT* versus *T* of metaphase chromosome suspensions of the Chinese hamster cell line DON. a) preparation in TAcCaM pH 3.2 (80 denaturation profiles); b) suspension in TAcCaM pH 7.0 (16 denaturation profiles). The frequency was weightened according to the relative peak intensity.

mamide treatment for 10 min, 2 hrs and 1 day. No difference was visible between chromosome suspensions without formamide treatment and 10 min formamide exposure. For 2 hrs formamide exposure, the frequency distribution around 80 °C appeared to be splitted into two distributions between 72 °C and 78 °C with a mean value of 74 °C (in this range typically no peak was found without formamide treatment) and between 80 °C and 86 °C with a mean value of 83 °C. This effect was intensified after a 1-day formamide exposure (Table I). One group of peaks was found between 70 °C and 74 °C (mean 71 °C), the other between 80 °C and 86 °C (mean 82 °C). In the latter case the gap between these two groups appeared to be more pronounced.

Discussion

In situ hybridization (ISH) is a routinely applied method to specifically label chromosomes by means of sequence-specific DNA probes. A prerequisite of most protocols is thermal denaturation in combination with formamide treatment (Lichter and Cremer, 1992). Recent publications showed that the denaturation behaviour of chromosomal targets and DNA probes can be studied photometrically. To measure the temperature dependent absorbance of both, suspensions *and* solutions, a high-resolution, computer controlled photometer with a vertical optical axis was designed and constructed (Wolf *et al.*, 1999; Bettag, 1989; Heiland, 1989). A computer program calculating the first derivative of absorbance was used to obtain a sample specific peak pattern in which thermally induced conformation changes can be represented by a typical peak at a certain temperature (Velten, 1989).

As expected from a theoretical approximation formula, a linear reduction of the DNA melting point was detected with increased formamide concentration. This principle behaviour was also found for DNA in TAcCaM, a chromosome isolation buffer (Wolf *et al.*, 1999, Schaller *et al.*, 1990) used in flow cytometry (Hausmann *et al.*, 1991; 1993; Hausmann and Cremer, 1998) to maintain the chromosomal morphology.

The experiments with metaphase chromosomes showed, compared to DNA, a completely different pattern of absorbance indicating that thermal denaturation may not be restricted only to DNA melting. But it has to be considered that the measurements presented here describe global denaturation effects and not the in situ denaturation conditions of *individual* chromosomes fixed on slides. In addition, according to the preparation protocol it cannnot be excluded that cell fragments were

still found in the suspension. These effects may both contribute to peak variabilities. At about 80 °C, the range of "complete denaturation" a typical peak was found in all measurements of both pH values accompanied by a "peakless" range between 70 °C and 77 °C.

In contrast to pure DNA, for metaphase chromosomes in suspension, 20% formamide treatment did not simply reduce the temperature of the range of "complete denaturation". Moreover, prolonged formamide treatment appeared to separate the range of "complete denaturation" into two clusters of conformation changes. This may suggest that formamide induces modifications on the metaphase chromosomes which may be denatured thermally at different temperatures. The lower peak group correlates to the usually applied denaturation temperatures for *in situ* hybridization at about 70 °C.

It appears to be reasonable to assume that the photometric data presented here may be not only valid for ISH of chromosomes in suspension (Dudin *et al.*, 1987; Hausmann *et al.*, 1991) but allow a useful hypothesis in general for ISH-procedures: One might draw the conclusion that in formamide protocols (e.g. Lichter and Cremer, 1992) using typically about 70 °C for DNA-probe *and* chromosomal target denaturation, a complete target denaturation might not always be achieved. In contrast to these protocols, recently developed non-formamide protocols (Durm *et al.*, 1996; Durm *et al.*, 1998; Haar *et al.*, 1996) use "complete" thermal denaturation up to 90 °C to 95 °C. However, in formamide ISH protocols, higher concentrations of formamide (typically 50–70%) and other chemical agents are involved in the denaturation process whereas in the present experiments, only 20% formamide was applied. Moreover, typically a pH > 7.0 is used. Thus, further investigations may be useful, where photometric and/or microscopic measurements are performed in closer correlation with ISH-experiments.

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