Denaturation behaviour of DNA-protein-complexes detected in situ in metaphase chromosomes in suspension by Hoechst 33258 fluorescence

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The denaturation behaviour of DNA-protein complexes in metaphase chromosomes in suspension was analysed in situ by Hoechst 33258 fluorescence. The results indicate that due to the stability of the dye molecule and the product of the molecular extinction coefficient and the quantum yield at different temperatures, Hoechst 33258 is a suitable probe for the detection of double-stranded DNA. Thus, it is possible to monitor the concentration of double-stranded DNA in a suspension by measuring the total fluorescence intensity. The fluorescence denaturation profiles of DNA (calf thymus) were found to be comparable to absorption measurements. The decrease in fluorescence of metaphase chromosomes in suspension with increasing temperature may therefore be used to detect conformational changes of DNA in situ.

1. Introduction

Methods to determine the denaturation behaviour of nucleic acids have been well established in biology since the early work of Marmur and Doty [1] (for a review, see ref. 2). Thermal denaturation is used routinely to determine the AT/GC ratio and quality of DNA samples photometrically. More complex structures, such as chromatin, are being investigated by a number of groups as reviewed by Li [3] and Van Holde [4]. Most of these works were performed with absorption measurements at 260 nm in order to determine hyperchromicity as a function of strand

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Abbreviations: HO, Hoechst 33258 (Bisbenzimid); AO, acridine orange; ds, double-stranded; ss, single-stranded; DAPI, 4,6-diamidino-2-phenylindole; ct, calf thymus.

separation. Another approach is to use probes with a characteristic behaviour after binding to dsDNA or ssDNA. An example of such probes is the fluorochrome acridine orange (AO), the fluorescence of which is bright green when bound to dsDNA and red when bound to ssDNA [5].

In many cases, however, fluorescence probes may have two major disadvantages:

(1) The quantum yield decreases with increasing temperature [6], or the fluorochromes are not stable at temperatures required for DNA denaturation. Thus, the temperature for adding the fluorochrome and performance of the photometric measurement must be fixed at 0°C, for instance. Because of the instability of the fluorochrome at higher temperatures, every measurement must be carried out with another aliquot of the sample. In metaphase chromosomes in suspension, however, very fast reassociation has been observed [7]; from this, it follows that it is not possible to conserve the conformational state adopted at a given temperature by quenching on ice.

(2) Ligands, especially AO, influence the structure of the DNA itself. At high concentrations, AO is known to denature dsDNA [8].

These disadvantages may be overcome by using HO as a fluorescence probe. HO appears to be physically stable for temperatures up to 100 °C; it does not exert a major influence on the DNA structure [9]. On this basis, we have developed a new approach to determine the denaturation behaviour of isolated metaphase chromosomes in suspension. The method leads to an excellent correlation between fluorescence and absorption measurements. To date, only a few reports are available on the 'denaturation' behaviour of chromosomes [10]; to our knowledge, no report has been presented on metaphase chromosomes in suspension. These are required for a broad spectrum of investigations in biology and medicine (for reviews, see refs 7, 11 and 12). A better understanding of their thermal behaviour may be interesting not only for theoretical reasons but may also contribute considerably to methodological progress [7,13–16].

2. Material and methods

DNA measurements were performed with commercially available ctDNA (type I, 'highly polymerized', Sigma, Deisenhofen, F.R.G.). For chromosome measurements, isolated metaphase chromosomes of cells of the Chinese hamster × human hybrid cell line GB3 (cells kindly provided by Dr Buys, Groningen) were used. In this cell line 18 Chinese hamster chromosomes and both human chromosomes 13 have been identified [17]. The hybrid cells were grown in 750-ml Falcon flasks in Ham's F-10 medium supplemented with 10% fetal calf serum, 1% penicillin/streptomycin solution (10000 U, 10000 μg/ml). Mitotic cells were synchronized by a Colcemid block of 5 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.

From an aliquot the amount of mitotic cells in the hypotonic solution was determined. After centrifugation (350 × g, 15 min) the hypotonic solution was removed, and 1 ml isolation buffer (25 mM Tris-acetic acid, 5 mM MgCl$_2$, 5 mM CaCl$_2$, pH 3.2) was added. In the same buffer, the mitotic cells were sonicated in a water bath (41 kHz, 30 W) to disrupt the cell membrane, and a suspension of dispersed chromosomes was obtained. The suspension can be stored at 4°C for several months without any visible morphological change. For the measurements, the ctDNA as well as the chromosomes were transferred to SSC (1 × SSC: 0.15 M NaCl, 0.015 M sodium citrate) at different concentrations: 0.1 × SSC, 1 × SSC and 2 × SSC.

The concentration of HO was determined by absorption measurements to 1 μM (final concentration of free dye). AO and DAPI were used at a concentration of about 1 μM. The thermal stability of the fluorescence emission by DAPI, AO and HO (free dye) was tested. Fluorescence of the drug warmed to 25°C and quenched on ice, $F^0(25)$, was compared to that of the drug after heating to 90°C (incubation for 15 min) and quenching on ice again $F^0(90)$. ΔF was defined as follows

$$\Delta F = F^0(90)/F^0(25) \times 100\%.$$

Under the condition of

$$F(T) \propto \epsilon(T)Q(T)c(T)$$

where $\epsilon(T)$ denotes the temperature-dependent molecular extinction coefficient, $Q(T)$ the temperature-dependent quantum yield and $c(T)$ the temperature-dependent concentration of unaffected dye. Less than 100% of ΔF indicates some irreversible change in the molecular structure of the dye.

As a measure of the change in the product of the quantum yield and the molecular extinction coefficient with temperature, the value of $\Delta(Q\epsilon)$ was determined:

$$\Delta(Q\epsilon) = \frac{F^{25}(25)}{F^{90}(90)/\Delta F} \times 100\%,$$

where $F^{25}(25)$ and $F^{90}(90)$ represent the fluorescence at 25 and 90 °C, respectively.

The (chromosomal) DNA was stained by addition of the fluorochrome to the suspension. The fluorescence of a sample ($\approx 10^5$ chromosomes or ≈ 1 μg ctDNA) and that of a reference were detected in a heated quartz cuvette using a fluorescence spectrometer (Perkin Elmer MPF 44 A). The excitation and emission wavelengths employed for HO-dsDNA complexes were 360 and 460 nm, for DAPI-dsDNA complexes 360 and 460 nm, and for AO-dsDNA complexes 490 and 530 nm [18]. As a reference the same concentration of buffer solution (SSC) including the fluorochrome was used as in the actual sample. Heating was carried out with a temperature-controlled water bath. The stability in temperature was better than 0.5 °C during a period of 15 min. The temperature of the suspension was recorded in the cuvette by a coaxial thermoelement and registered on a compensated thermograph (Goertz Metrawatt Servogor 460). For each fluorescence measurement, 1 ml sample and 1 ml reference were used. DNA was measured immediately after attainment of the equilibrium temperature. The chromosomes were measured after 15 min of incubation at the given temperature. Two different kinds of experiments were performed: one separate aliquot of a sample was used for each temperature measured; only one aliquot was used for all temperatures measured.

The relative fluorescence F of the sample was determined by normalizing to the reference according to the following formula:

$$F = \frac{F_s - F_{ref}}{F_{ref}}.$$

where F_s denotes the fluorescence of the sample and F_{ref} corresponds to that of the reference, i.e., the buffer with the fluorochrome only.

To measure the absorption of suspensions and solutions as a function of temperature, a high-precision photometrical system has been developed (U. Bettag et al., manuscript in preparation). This device has the following characteristics: mercury lamp, double-beam system with optical feedback (PTI, Tornesch, F.R.G.); detection with a photomultiplier tube in a cooling house (Thorn EMI,

U.K.); vertical direction of the beam for suspension measurements; resolution of temperature about 10^{-3} °C; minimum peak-to-peak distance in derivative hyperchromicity curves about 0.5 °C; significant peaks of $d(\Delta A)$ are peaks with $d(\Delta A) > 0.0005$ [°C]$^{-1}$.

The absorption A is defined as the logarithm ($\log_{10}$) of the ratio of the light intensity passing the reference $I_{ref}(T)$ and that passing through the sample $I_s(T)$. Here, ΔA is defined as the difference between the absorption at the actual temperature T and the initial temperature T_0

$$\Delta A = \log_{10} \frac{I_{ref}(T)}{I_s(T)} - \log_{10} \frac{I_{ref}(T_0)}{I_s(T_0)}.$$

3. Results

3.1. Thermal stability of the fluorescence

The thermal stability of the fluorescence of DAPI, AO and HO (free dye) was investigated as described above. Table 1 lists the values of ΔF and $\Delta(Q\epsilon)$ for the three fluorochromes. Obviously, the ΔF value obtained for HO was not significantly different from unity (100 $\pm 3\%$). This signifies that the fluorescence of HO was the same (or almost the same) at both temperatures, suggesting that the fluorochrome was not affected by the thermal treatment. The same result was observed on heating HO for over 3 h. The ΔF values determined for DAPI and AO, however, were

Table 1

Thermal stability of fluorescence properties

$\Delta F = [F^0(90)/F^0(25)] \times 100\%$ characterizes the stability of the fluorochrome itself; $\Delta(Q\epsilon) = [F^{25}(25)/\{F^{90}(90)/\Delta F\}] \times 100\%$ expresses the stability of the fluorescence quantum yield and molecular extinction coefficient. Data are mean values and standard deviations of seven independent measurements each.

Fluorochrome	ΔF (%)	$\Delta(Q\epsilon)$ (%)
DAPI(λ_{em} 460 nm)	50 ± 4	85 ± 7
AO(λ_{em} 530 nm)	57 ± 2	200 ± 30
HO(λ_{em} 460 nm)	100 ± 3	100 ± 10

considerably lower than 100%. This reduction might be due to an irreversible change in their molecular structure.

The temperature dependence of $\Delta(Q\epsilon)$ was found to be weak for HO. This was not the case for DAPI and AO, which both showed a considerable temperature dependence of $\Delta(Q\epsilon)$. Furthermore, at the HO concentration used, the fluorescence of HO was proportional to the concentration of dsDNA and chromosomes measured and compared at the same temperature [12]. Additional measurements with HO suggested that unspecific binding to histones was negligible, since no change in fluorescence in comparison to free dye was observed in the presence of histones (data not shown).

3.2. Comparison of HO fluorescence and absorption measurements for studies of the denaturation behaviour

As described in section 2, measurements of isolated metaphase chromosomes (or DNA) were performed in two ways. No significant difference was observed. Therefore, all subsequent measurements were performed with only one aliquot for all temperatures measured.

Fig. 1 shows results obtained for HO fluorescence and absorption measurements of high resolution of ctDNA dissolved in 1 × SSC.

The denaturation ('melting') profiles of fig. 1a were detected by ultraviolet absorption. The absorption (curve ΔA) and its first derivative (curve $d(\Delta A)$) are shown as a function of temperature. Six peaks (nos 1–5) of the $d(\Delta A)$ curve were assigned in the range of 75–90°C.

Fig. 1b shows a fluorescence profile for HO of an aliquot of the DNA sample used in the absorption measurements of fig. 1a. Curve F shows the dependence of HO fluorescence on temperature (spline interpolation of the experimental data [19]). From curve F, the opposite of the first derivative (curve dF) was calculated; again six peaks were observed in the range from 75 to 90°C. For the measurement shown in fig. 1b, 60 experimental points were registered. Ten additional independent measurements were made at lower resolution (10 experimental points). In these cases the individual peaks nos 1–5 merged into one broad peak.
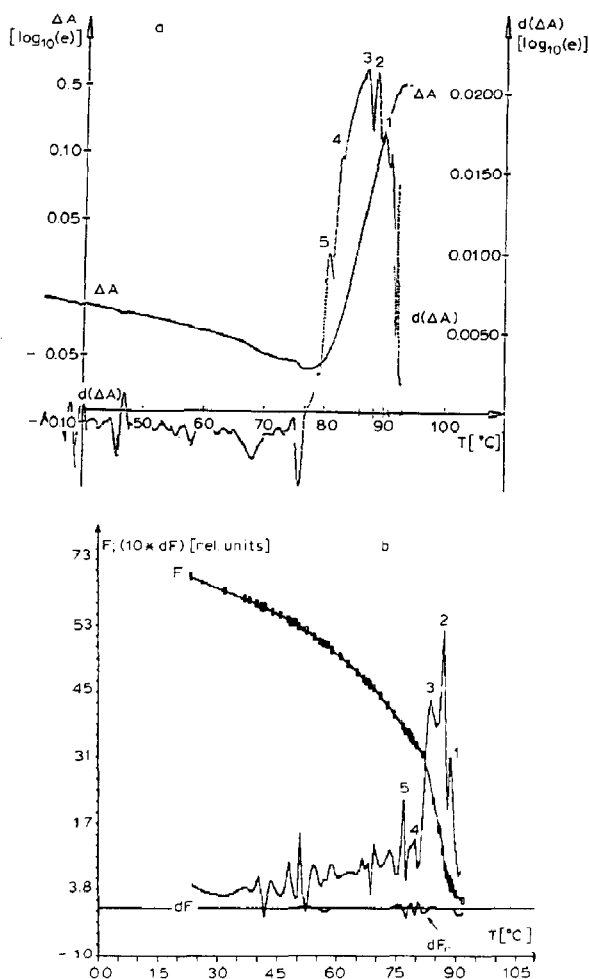

Fig. 1. 'Denaturation profiles' of calf thymus DNA in 1 × SSC. (a) ΔA and the first derivative ($d(\Delta A)$) on the ordinate (both in units of $\log_{10}(e)$) vs temperature (in °C) on the abscissa. ΔA represents the difference between the absorption at the actual temperature T and the initial temperature T_0. The data were interpolated by a polynomial fitting curve. (b) Fluorescence (F; in arbitrary units) and the opposite of the first derivative (dF; magnification 10×) on the ordinate vs temperature (in °C) on the abscissa. The data (□) were interpolated by a spline function [18]. For comparison, the opposite of the first derivative (dF_r) of a control measurement is shown; here the reference was measured against an aliquot of the same solution. The maxima of the derivatives at temperatures above 75°C are numbered for comparison.

A reasonable linear correlation ($r = 0.95$) was obtained between $T_{d(\Delta A)}$ (peak temperature obtained from absorption measurements; fig. 1a)

Table 2

Comparison of the main transition points of the denaturation profiles of ctDNA at two salt concentrations (SSC)

T_f, temperature at the absolute maximum of the opposite of the first derivative of the fluorescence curve; T_m, temperature at the absolute maximum of the first derivative of the absorption curve.

SSC	T_f ($^\circ$C) [a]	T_m ($^\circ$C) [b]	T_m ($^\circ$C) [c]
0.1×	70.4 ± 4.0	71.0 ± 1.5	70.4 ± 2.0
1.0×	87.6 ± 2.0	87.0 ± 1.5	87.0 ± 2.0

[a] T_f was determined by fluorescence measurements (cf. fig. 1b).
[b] T_m was determined by absorption measurements (cf. fig. 1a).
[c] Data were calculated by linear extrapolation of results from ref. 17 to a T_m 87 $^\circ$C and 1 × SSC.

and T_{dF} (peak temperature obtained from fluorescence measurements; fig. 1b). For comparison with data from the literature, a salt concentration of 0.1 × SSC and 1 × SSC of the buffer was used; the main transition point (T_f) of the fluorescence profile of ctDNA was determined, i.e., the temperature of the absolute maximum of the opposite of the first derivative of the fluorescence curve. In addition, the corresponding main transition points (T_m), here defined as the centre of maxima of the curve $d(\Delta A)$, of the absorption profiles (between 75 and 95 $^\circ$C) were measured. Further data were calculated by linear extrapolation using results from the literature [20]. The results are listed in table 2. At the same SSC concentration, the T_f values are in excellent agreement both with the corresponding T_m values determined from independent absorption measurements, and with those taken from the literature.

3.3. Temperature-dependent fluorescence denaturation profile of chromosomes in suspension

The fluorescence approach described above can be used not only for DNA measurements, but also for isolated metaphase chromosomes in suspension. The fluorescence signals obtained from DNA and chromosomes showed a different behaviour vs time after reaching equilibrium temperature. The time needed to give rise to a stable fluorescence signal for chromosomes was up to 10 min, whereas the fluorescence signal of DNA attained stability
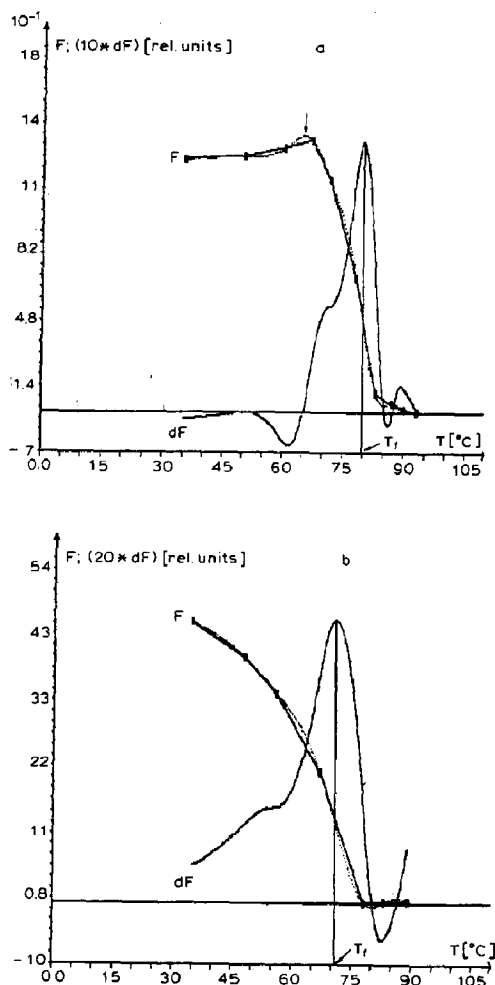

Fig. 2. Fluorescence profiles of metaphase chromosomes and DNA in 0.1×SSC. (a) Fluorescence (F) of chromosomes of the Chinese hamster × human hybrid cell line GB3 (in arbitrary units) and the opposite of the first derivative (dF; magnification 10×) on the ordinate vs temperature (in $^\circ$C) on the abscissa. The data (□) were interpolated by a spline function. The arrow (↓) indicates a significant peak at low temperature (62 $^\circ$C) only visible in the fluorescence profiles of chromosomes. The temperature T_f of the main transition point (absolute maximum of the opposite of the first derivative) is indicated by a vertical line (T_f = 79.9 $^\circ$C). (b) Fluorescence (F) of ctDNA (in arbitrary units) and the opposite of the first derivative (dF; magnification 20×) on the ordinate vs temperature (in $^\circ$C) on the abscissa. The data (□) were interpolated by a spline function. The temperature T_f of the main transition point (absolute maximum of the opposite of the first derivative) is indicated by a vertical line (T_f = 70.4 $^\circ$C).

after a shorter period of time (< 1 min).

Fig. 2a shows the fluorescence profile of isolated chromosomes of a Chinese hamster × human hybrid line (GB3). As in fig. 1b, the spline interpolation (F) and the opposite of the first derivative (dF) are given. For the purpose of comparison, fig. 2b shows the fluorescence profile of ctDNA of a similar resolution.

The main transition point T_f was observed to be 70.4°C for ctDNA and 79.9°C for GB3 chromosomes. In fig. 2a, a plateau was observed between 30 and 50°C; furthermore, a characteristic peak (arrow) was identified. Both observations were not detected by measurements with pure ctDNA (figs 1b, 2b and 15 other independent measurements with DNA and chromosomes, respectively).

4. Discussion

Hoechst 33258 (HO) is a fluorochrome displaying considerable stability of the fluorescence at different temperatures (table 1). Since the binding of HO is highly specific to dsDNA [21,22], HO can be used to monitor dsDNA at different temperatures.

Using merely a single aliquot of a sample for all temperatures per measurement, only a small quantity of material will be required. Therefore, routine analysis even with limited quantities of material becomes possible.

The fluorescence profile of DNA was found to be fully compatible with the denaturation profiles determined by absorption (260 nm) techniques, as shown in fig. 1 and table 2.

The results indicate that, by measuring HO fluorescence, it is possible to study in situ the temperature-dependent denaturation behaviour of metaphase chromosomes in suspension (fig. 2a). Furthermore, it may also become possible by this method to investigate the temperature-dependent behaviour of chromatin in cell nuclei in suspension.

Reassociation of chromosomal DNA (as measured by fluorescence) may occur within a rather short time [7]. On the other hand, we observed that at a given temperature the fluorescence signal of chromosomes became stable only after 10 min,

while this was achieved within less than 1 min for DNA. Thus, it is not possible to fix the state of denaturation in metaphase chromosomes achieved at a given temperature by quenching the sample on ice at different temperatures. Fluorescence probes with a temperature-dependent quantum yield and molecular extinction coefficient or probes which are unstable at temperatures near 100°C are unsuitable to detect temperature-dependent behaviour of DNA. With HO such difficulties can be overcome and thus it is possible to measure even the same sample.

The DNA of GB3 chromosomes may be compared with pure Chinese hamster DNA, since the AT/GC ratio of human chromosome 13 is about the same as that of the Chinese hamster DNA [23]. Since ctDNA (58% AT, according to the manufacturer's specification) and Chinese hamster DNA (about 60% AT) [24] do not vary greatly in base ratio, a comparison at this level of resolution may be reasonable under the assumption that the binding behaviour of Hoechst 33258 is similar in the case of chromosomal DNA and of free DNA.

A comparison of the fluorescence profiles of metaphase chromosomes of a Chinese hamster × human hybrid cell line with ctDNA shows that the main transition point T_f of DNA in the chromosomes ($T_F = 79.9°C$; fig. 2a) is shifted to higher temperatures compared with pure DNA ($T_f = 70.4°C$); fig. 2b). Such an increase is compatible with the notion of the confinement of structural proteins on the DNA.

The decrease in fluorescence in the premelting region of isolated DNA was not observed with chromosomes (15 independent experiments under the same conditions). This decrease might be due to structural changes in the DNA.

Under the conditions used, chromosomes showed a characteristic increase in fluorescence at a temperature of about 60°C (fig. 2a; arrow). It may be speculated that at this temperature a number of protein binding sites are removed from DNA. These binding sites may then be occupied by additional HO molecules.

The approach described here introduces the possibility of probing the denaturation behaviour of metaphase chromosomes in suspension even for a small amount (< 10^6 chromosomes) of material.

The method may be also applicable to suspended nuclei without modifications. Thus, it may become possible to optimize procedures for particular conditions, e.g., antibody binding against BrdUdr [25] or in situ hybridization in suspension to isolated chromosomes [7,13–16] or cell nuclei [26,27].

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