

Unfolding and Double-stranded DNA Binding of the Cold Shock Protein Homologue Cla h 8 from *Cladosporium herbarum**

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The cloning, purification, and biophysical characterization of the first eukaryotic cold shock protein homologue, Cla h 8, expressed as single functional polypeptide is reported here. It was discovered as a minor allergen of the mold *Cladosporium herbarum* by phage display using a library selectively enriched for IgE-binding proteins. Based on the sequence homology of Cla h 8 with bacterial cold shock proteins (CSPs), a homology-based computer model of the allergen was computed indicating an all- β structure of Cla h 8. This major structural feature was confirmed by CD spectroscopy. Despite the structural similarities with bacterial CSPs, the DNA-binding and unfolding behavior of Cla h 8 exhibited unique and previously undescribed characteristics. High affinities of Cla h 8 for single-stranded DNA as well as for double-stranded DNA corresponding to the human Y-box were detected. The affinity for double-stranded DNA increased significantly with decreasing temperature, which was paralleled by an increase in the β sheet content of the protein. Temperature-dependent fluorescence anisotropy and far-UV CD measurements revealed different unfolding transitions at 28 and at 35.7 °C, respectively, indicating a multistate transition, which is uncommon for CSPs. The enhanced affinity for DNA at low temperatures together with the low unfolding transition refer to the functional significance of Cla h 8 at reduced temperatures.

Cold shock proteins (CSPs)¹ are a class of small proteins which occur in a variety of prokaryotic organisms like mesophilic, psychrotrophic, and thermophilic bacteria (1, 2). Structurally, they belong to the oligonucleotide/oligosaccharide binding-fold family (3), which comprise a five-stranded β -barrel-fold and contain the highly conserved ribonucleoprotein consensus sequences RNP1 and RNP2 (4–8). Exposed aromatic residues on these motifs are crucial not only for nucleotide binding but also for stability and folding of CSPs (9–12). Initially, it was thought that prokaryotic CSPs bind preferentially to the sin-

gle-stranded ATTGG Y-box motif, in analogy to Y-box-binding proteins (Yb proteins) (7, 13–15), but in recent work it has turned out that binding to ssDNA templates is not limited to just this sequence, and that it depends on the T-base composition of the strand (16–18).

CspA from *Escherichia coli*, the first known member of this protein class, was shown to be dramatically overproduced at low temperature stress (19, 20), and thus the entire protein family was attributed a cold shock regulatory function (1, 2). CspA acts as an RNA chaperone (21) in the course of translational control at decreased temperatures, binding nonspecifically to RNA in order to prevent the formation of secondary structure and thereby enabling efficient initiation of translation. Further, it regulates the synthesis of other cold stress-inducible proteins through transcription antitermination (22). However, evidence has arisen that the physiological roles of cold shock proteins are not limited to responses at low temperatures (1, 2). Many of them are expressed under physiological (growth) conditions, and it has been recently demonstrated that the production of CspA is also induced at 37 °C upon nutritional upshifts (23).

In eukaryotes, cold shock proteins are present as functional domains of Yb proteins (24). They were first reported to bind to the inverted CCAAT box (Y-box) of the major histocompatibility complex class II promoter sequence (13). Yb proteins consist of a glycine-rich N-terminal domain with unknown function, a nucleic-acid binding (cold shock) domain, and a C-terminal domain responsible for dimerization (27). They are multifunctional regulators of gene expression, and they are presumed to couple transcription to translation of mRNA (24, 25). In addition to their binding of RNA, Yb proteins were shown to be able to interact with double-stranded DNA (14, 26, 27), while bacterial CSPs were found to interact only with single-stranded but not with double-stranded DNA (4, 15, 17).

Here we report the biophysical characteristics of the first eukaryotic cold shock protein, which was found to be independently expressed as functional polypeptide and not as part of a Yb protein. The cold shock protein homologue was named Cla h 8 and is a minor allergen of *Cladosporium herbarum* (28). Allergic reactions to this mold lead to rhinitis, conjunctivitis, and allergic asthma. Although Cla h 8 shows high homology to bacterial cold shock proteins, it was found to display some previously unobserved characteristics especially with respect to DNA binding. It was shown to unfold at temperatures uncommonly low for CSPs, which is interpreted to correlate with the improved affinity of Cla h 8 for dsDNA at low temperatures referring to the functional significance of Cla h 8 at reduced temperatures.

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¹ The abbreviations used are: CSP, cold shock protein; Yb proteins, Y-box binding proteins; ss, single stranded; ds, double-stranded; CspA, cold shock protein A from *E. coli*; CspB, cold shock protein B from *B. subtilis*; Cla h 8, cold shock protein from *C. herbarum*; CD, circular dichroism.

MATERIALS AND METHODS

Construction and Screening of a *C. herbarum* cDNA Library Displayed on Phage Surface—For the construction of a *C. herbarum* cDNA library, 4 μ g of a modified pJuFo vector (29) was digested with *Eco*RI and *Xho*I and subsequently treated with calf intestine phosphatase (30). 2 μ g of *C. herbarum* cDNA inserts, prepared from a previously constructed λ -Zap expression library (28) by *Eco*RI and *Xho*I digestion of isolated pBlueScript phagemid DNA excised *in vivo* (31), was ligated into the cleaved vector. The ligation mixture was used to transform *E. coli* XL1-Blue cells by electroporation, and an M13 phage surface expression library was generated by helper phage superinfection as previously described (32). The library was selectively enriched for phage displaying IgE-binding proteins by biopanning in microtiter plates coated with pooled serum IgE from individuals allergic to *C. herbarum* captured with monoclonal mouse anti-human IgE monoclonal antibody TN-142 (33). Serum donors were selected according to case history, specific IgE to *C. herbarum* determined by radioallergosorbent test and skin reactivity to commercial *C. herbarum* extracts (34). A wide variety of phage binding to human serum IgE was isolated.

Identification of a Clone Encoding Cla h 8—Enriched phagemids were used to infect *E. coli* XL1-Blue cells, plated onto square agar plates at a density of 2000–5000 colonies per plate and grown at 37 °C overnight. Using a picking/gridding robot, 5376 colonies were arrayed into 384-well microtiter plates, grown at 37 °C, and replicated into new microtiter plates using a 384-pin replication tool to produce working copies (35). cDNA inserts of all picked clones were amplified by PCR in 384-well format, and PCR products were gridded in duplicate onto 222 $\times$ 222 mm Nylon filter membranes at a density of 27,648 spots/filter using a picking/gridding robot (36). Repetitive filter hybridizations using digoxigenin-labeled PCR probes derived from randomly selected clones were performed as described (37) until all different sequences contained in the 5376-clone sample were identified (38). The 38 different inserts obtained were sequenced using the dideoxynucleotide chain termination method (39). Both DNA strands were sequenced using vector-derived primers. Homology searches and sequence comparisons were performed with BLAST and the Genetics Computer Group program FASTA. One clone revealed strong homology with nucleotide sequences encoding cold shock protein.

Cloning, Expression, and Purification of Recombinant Cla h 8—The cDNA encoding Cla h 8 was amplified by standard PCR from the corresponding phagemid using the following primers: 5'-primer, 5'-CG GGA TCC ATG GAC GCC TCC ACC GAA CG-3'; 3'-primer, 5'-CC AAG CTT TTA GTT GTT GCG GAC GCT GG-3', thus introducing a *Bam*HI and a *Hind*III restriction site (*underlined*). PCR cycling conditions were 94 °C for 60 s, 54 °C for 60 s, and 72 °C for 60 s for 30 cycles, followed by a terminal extension cycle at 72 °C for 10 min. The amplification product was purified over QIAquick spin columns (Qiagen Inc., Chatsworth, CA), digested with *Bam*HI and *Hind*III, ligated to *Bam*HI/*Hind*III-restricted p(His)₆-DHFR vector (40) and transformed into *E. coli* strain M15 (41) by electroporation. Transformants were grown in liquid culture to verify the nucleotide sequence (30) and used to produce hexahistidine-tagged recombinant protein. The specific binding of human serum IgE from individuals sensitized to *C. herbarum* to recombinant Cla h 8 was analyzed by an antigen-specific enzyme-linked immunosorbent assay as described (42).

A single colony containing M15 *E. coli* cells was inoculated in LB Amp/Kana medium at 37 °C to an optical density of 0.5–0.9. The expression of His₆-Cla h 8 was induced by the addition of 1 mM isopropyl-1-thio- β -D-galactopyranoside. After 5 h, the cells were harvested by centrifugation (15 min, 5000 $\times$ g), and the pellet was resuspended in extraction buffer (50 mM NaP_i, pH 7, 300 mM NaCl, 0.1% Trasyolol). Subsequently, the cells were broken by ultrasonication (3 $\times$ 20 s, 80 W) on ice followed by centrifugation at 10,000 $\times$ g for 20 min. The supernatant was tested by SDS-PAGE for the presence of soluble His₆-Cla h 8 fusion protein and was then applied to a TALON metal affinity resin column (CLONTECH Laboratories, Palo Alto, CA). The His₆-Cla h 8 fusion protein was eluted from the resin by pulsing with 1 ml of elution buffer (50 mM NaP_i, pH 7, 300 mM NaCl, 0.1% Trasyolol, 150 mM imidazole). After this step, a purification of 95% was achieved. To further increase the purity of the protein, the combined Cla h 8-containing fractions were dialyzed against buffer A (20 mM NaP_i, pH 7.2) and loaded onto a Q-Sepharose column (Amersham Biosciences). A discontinuous gradient was run from 0 $\rightarrow$ 60% B in 10 min, and from 60% $\rightarrow$ 100% B in 50 min (buffer B = 20 mM NaP_i, 1.2 M NaCl, pH 7.2). Cla h 8 with a purity of >99% was thus obtained (see Fig. 1). Fractions containing pure Cla h 8 were pooled, extensively dialyzed against 50 mM NaPi and 50 mM NaCl, pH 7.2, and stored at -20 °C. The concen-

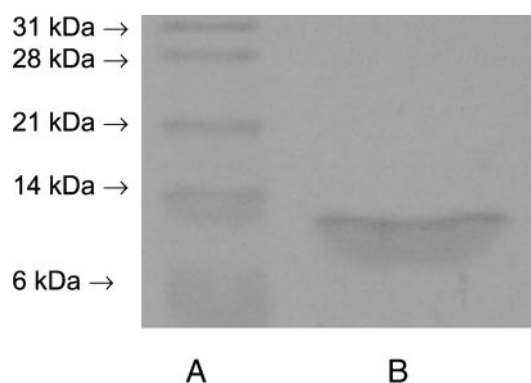


FIG. 1. SDS-PAGE of purified Cla h 8 after Coomassie Blue staining. Lane A, molecular mass marker. Lane B, Cla h 8.

tration of Cla h 8 was determined by the Bradford assay (43).

CD Measurements—The CD spectrum of 5 μ M Cla h 8 was recorded on a Jasco J-710 spectropolarimeter (Japan Spectroscopic, Tokyo, Japan). Far-UV CD measurements were carried out in the range between 195–250 nm using a quartz cuvette with a path length of 0.1 cm, a response time of 0.25 s, and a data point resolution of 0.1 nm. Three scans were recorded to obtain smooth spectra. Mean residue ellipticities of background-corrected spectra were calculated by JASCO standard analysis. Analysis of the spectra with respect to secondary structure content was performed using the algorithm SELCON (44). For thermal denaturation studies, Cla h 8 was diluted into 50 mM NaP_i, pH 7.2, and 50 mM NaCl to a final concentration of 38 μ M. A spectrum at the indicated temperature was recorded after an equilibration period of 3 min.

Homology Modeling of Cla h 8—A model of the three-dimensional structure of Cla h 8 was obtained by homology-based modeling using the Swiss-Model server (45). As templates, the structures of the CSPs of *E. coli*, *Bacillus subtilis*, and *Bacillus caldolyticus* (PDB entries 3MEF, 1NMG, and 1C9O) were used. Further refinement of the Cla h 8 structure was accomplished by applying 200 steps of steepest descent energy minimization followed by a 100-ps molecular dynamics simulation using the program Insight II and Discover (Molecular Simulation Inc., San Diego, CA). Throughout the refinement procedure, the total energy of the system was found to decrease and to reach a stable minimum value after 30 ps of molecular dynamics simulation.

Isothermal Fluorescence Titrations—Steady state fluorescence measurements were performed at room temperature on a Perkin Elmer Life Sciences LS50B fluorometer. The emission of a 60 nM equilibrated Cla h 8 solution upon excitation at 282 nm was recorded over the range of 300–400 nm. Binding isotherms were obtained by coupling the cuvette holder to a thermostat and by the addition of an aliquot of the respective oligonucleotide ligand, allowing for an equilibration period of 2 min. The slit width was set at 10 and 15 nm for excitation and emission, respectively, and the spectra were recorded with 200 nm/min. A 290-nm cut-off was inserted to avoid stray light. As ss ligands, the oligonucleotides 5'-GTGGGAATCCTACTGATTGGCCAAGGTGCTGGTGG-3' (Yb⁺) and 5'-CCACCAGCACCTTGGCCAATCCAGTAGGATTCCCA-C-3' (Yb⁻) were used. The concentration of the oligonucleotides was determined by their absorption at 260 nm. To obtain the corresponding dsDNA (Yb $\pm$), equimolar amounts of Yb⁺ and Yb⁻ were annealed by heating the mixture for 5 min at 65 °C followed by a slow cooling period of 60 min to 30 °C. According to reversed phase high performance liquid chromatography (data not shown), the yield of dsDNA thus obtained was >95% Yb $\pm$, which was therefore used in the isothermal titration experiments without further purification.

The fluorescence spectra of Cla h 8 were background-corrected, and the respective areas were integrated between 300 and 400 nm. The normalized mean changes in fluorescence intensity ($-\Delta F/F_0$) resulting from three independent experiments were plotted against the ligand concentration. The resulting binding isotherms were analyzed by non-linear regression using the program Origin (Microcal Inc.) to Equation 1 describing a bimolecular association reaction as described previously (17, 18, 46, 47)

$$F = F_i + F_{\max} \frac{K_d + [\text{Cla h 8}] + [\text{DNA}] - \sqrt{(K_d + [\text{Cla h 8}] + [\text{DNA}])^2 - 4[\text{Cla h 8}][\text{DNA}]}}{2[\text{Cla h 8}]}$$

(Eq. 1)

		β_1	β_2	β_3	β_4	β_5	
Cla h 8 <i>Cladosporium herbarum</i>	1	MDASTERQNGTVKWFNDEKGYGFITPENGSA	DLFVHFRAIEKDG	---	FKSLKEGEAVTFVAEQGQKGMQASSVRNN		73
TapB <i>Pseudomonas fragi</i>	1	MSQRQSGTVKWFNDEKGFIFITPESGP	-DLFVHFRAIQNG	---	FKSLKEGQKVTFIAVQGQKGMQA		63
cspA <i>Escherichia coli</i>	1	MSGKMTGIVKWFNADKGFGITPDDGSKDVFVHFS	AIQNDG	---	YKSLDEGQKVSTIESGAKGPAAGNVTS		70
cspB <i>Bacillus subtilis</i>	1	MLEGKVKWFNSEKGFIEVE	-GQDDVFVHFS	AIQGE	---	FKTLEEGQAVSFEIVEGNRGPQAANVTKEA	67
csp <i>Bacillus caldolyticus</i>	1	MQRGKVKWFNNEKGYGFIIEVEGGS	-DVFVHFTAQGE	---	FKTLEEGQEVSEIVEVQGNRGPQAANVVKL		63
CSD human Yb-1	61	GTVKWFNVNRNGYGINRNDTKEDVFVHQTAKKNNPRKYLRSVGDGETVF	EDVVEGEKGEAAV				125

FIG. 2. Sequence alignment of Cla h 8 with bacterial CSPs and the CSD of Yb-1. The predicted β -sheets of Cla h 8 are indicated based on the homology with the bacterial CSPs.

where F_i is the initial and $F_{\max}$ is the maximum fluorescence value, K_d is the dissociation constant, and [Cla h 8] and [DNA] are the total concentrations of Cla h 8 and DNA ligand, respectively. The fitted parameters were $F_{\max}$ and K_d .

Fluorescence Anisotropy—Temperature-induced anisotropy changes were recorded at an emission wavelength of 336 nm by a stepwise increase of the temperature of a 300 nM Cla h 8 solution in the range 8–90 °C. Five minutes of equilibration at each temperature were allowed before the anisotropy signal was measured over 30 s. The data shown represent the mean of three independent measurements.

RESULTS

Cla h 8 consists of 73 amino acids with a calculated molecular mass of 8.1 kDa. The sequence of Cla h 8 is shown in Fig. 2. It displays high sequence homology with bacterial CSPs like CspA from *E. coli* (76% homology) and CspB from *B. subtilis* (70% homology). Although from eukaryotic origin, it exhibits only 57% homology with the cold shock domain of the human Yb-binding protein 1. As in the bacterial CSPs, highly conserved regions within Cla h 8 are the β_1 -, β_2 -, and β_3 -sheets, which correspond to the putative oligonucleotide binding sites of the protein. Based upon the high sequence homology, a similar overall fold of Cla h 8 compared with the structurally well documented bacterial CSPs can be expected. It was therefore possible to compute a homology-based model of Cla h 8 based on the three-dimensional structures of the CSPs of *E. coli*, *B. subtilis*, and *B. caldolyticus* (4–7, 48) using for a first approach the Swiss-Model protein modeling server (45). The obtained structure was further refined by applying energy minimization and molecular dynamics computer simulation (see “Materials and Methods”). The final model of Cla h 8 exhibited a five-stranded β -barrel structure (not shown) and was therefore found to correspond to the general fold of the CSP family. However, the subtle differences in the primary structure between Cla h 8 and bacterial CSPs, e.g. an arginine residue at position 38 of Cla h 8, which is occupied by a serine or a threonine side chain in most bacterial CSPs, may be responsible for the differences in the oligonucleotide binding behavior outlined below.

To experimentally determine the secondary structure content of Cla h 8, far-UV CD spectra were recorded (see Fig. 3). Analysis of the spectrum recorded at 25 °C with respect to secondary structure (43) showed that 41% of the residues exhibit β -sheet structure. This value increased to 52% when the temperature was decreased to 15 °C (see Fig. 3) referring to an overall stabilization of the predicted all- β structure of Cla h 8 at reduced temperatures. Absolute values of secondary structure content must be regarded very cautiously because of the possible impact of aromatic amino acids on the secondary structure analysis especially in the β -sheet range. However, since we are comparing two states of the same protein under different conditions with respect to β -sheet content, the relative increase is revealed with high confidence. The α -helical content of Cla h 8 was found to be very low (<7%), independent from the temperature, thus further confirming the all- β structure of the protein.

Because of the structural stabilization of Cla h 8 observed at reduced temperatures (see Fig. 3), thermal unfolding curves using far-UV CD spectroscopy, which relate to the protein's

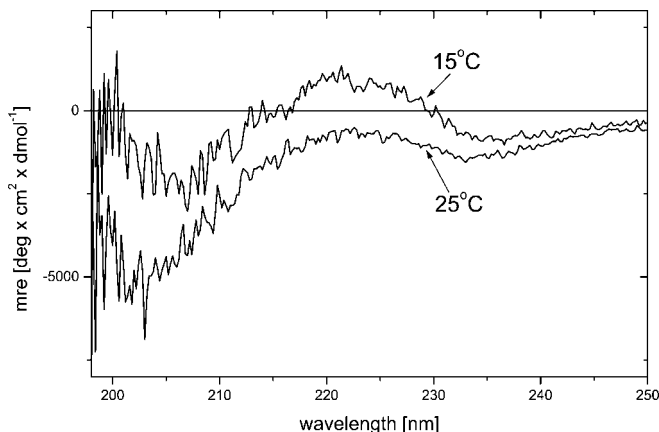


FIG. 3. CD spectra of 5 μ M Cla h 8 at 13 °C and at 25 °C. Shown are the mean residue ellipticities of the spectra averaged over three scans.

secondary structure, were recorded at three different wavelengths: 208 nm, 216 nm, and 222 nm. The results are displayed in Fig. 4. They reveal a transition of unfolding at 35.7 °C, which is uncommonly low compared with other bacterial CSPs (9–12). The transition of unfolding increased, however, to 42.2 °C upon addition of Yb+ (see Fig. 5), which is a ssDNA ligand of Cla h 8 (see below). Ligand binding obviously stabilizes the protein indicating an induced fit of Cla h 8 upon ligand binding.

To monitor the unfolding of Cla h 8 by detecting changes of the tertiary/quaternary structure, the temperature-dependent fluorescence anisotropy of the protein was recorded (see Fig. 6). The fluorescence emission of Cla h 8 was dominated by the intrinsic tryptophan chromophore at position 14. Depolarization of the fluorescence occurs due to the local movements of the fluorophore and to the rotational motion of the protein. The latter parameter corresponds to the hydrodynamic volume and thus to the oligomerization state of the macromolecule. As can be seen in Fig. 6, a single transition at 28.0 °C to lower anisotropy values upon increasing the temperature was obtained. The difference in the unfolding transitions observed in the CD and the fluorescence measurements clearly indicate a multi-state transition (51).

To investigate the affinity of Cla h 8 for DNA, isothermal fluorescence titration curves of Cla h 8 were recorded using, as in the fluorescence anisotropy measurements, the intrinsic fluorescence emission of Trp-14. This residue is highly conserved in the CSP family and is supposed to directly interact with oligonucleotides. As ligands, two ssDNAs were used, corresponding to the 35-mers of the sense (Yb+) and the antisense (Yb-) region of the human Y-box 1 (see “Materials and Methods”), as well as the annealed ds oligonucleotide Yb±. Unlike CspA and CspB (16–18), Yb- bound to Cla h 8 with a significantly higher affinity than Yb+ at the cellular growth optimum temperature (25 °C, see Table I). This clear discrimination was lost under cold shock conditions (13 °C). The β -sheet content, as detected by CD spectroscopy, was found to increase by the addition of a molar excess of each of the three ligands at 13 °C

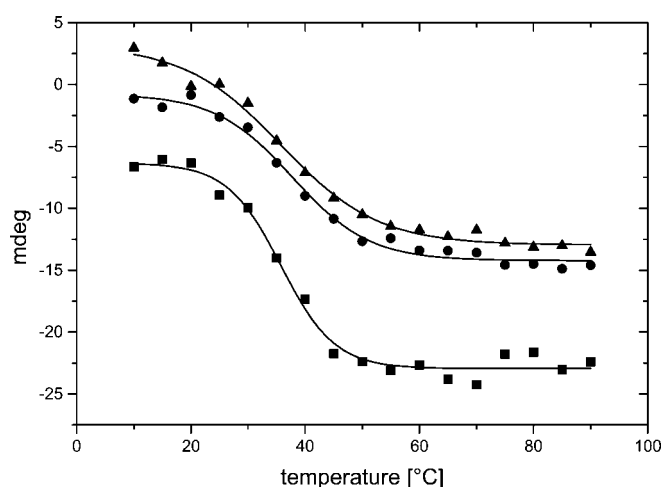


FIG. 4. Thermal unfolding of 38 μ M Cla h 8 monitored by far-UV CD spectroscopy at the wavelengths 208 (■), 216 (●), and 222 nm (▲). The values shown represent the mean values of three independent measurements. The transition point was calculated by fitting the data to the Boltzmann equation.

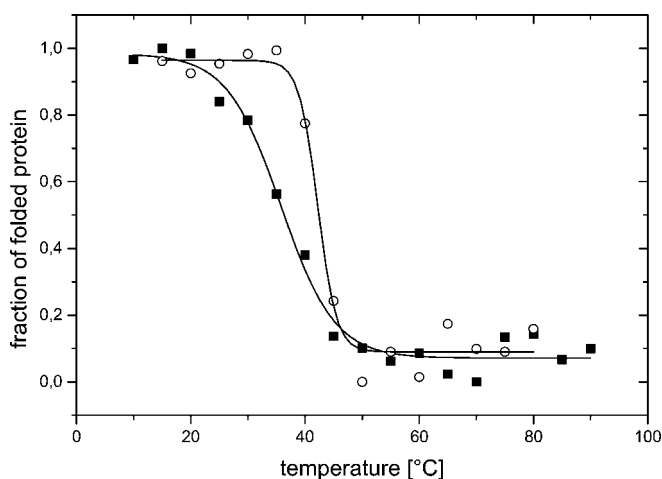


FIG. 5. Thermal unfolding of 38 μ M Cla h 8 monitored by far-UV CD spectroscopy at 208 nm in the absence (■) and in the presence (○) of the ssDNA ligand Yb+. The values shown represent the mean values of three independent measurements. The transition point was calculated by fitting the data to the Boltzmann equation.

(data not shown). Interestingly, at 13 °C as well as at 25 °C, only Yb[−] induced a 10-nm red shift of the fluorescence maximum upon complete saturation, whereas Yb⁺ and Yb[±] only quenched the fluorescence emission without a shift. This refers to different Cla h 8 binding geometries of Yb[−] compared with Yb⁺ and Yb[±]. Contrary to CspA, the dsDNA (Yb[±]) was found to bind with significant affinity to Cla h 8 at 25 °C (see Table I). This affinity was increased by a factor of 3–4 when the temperature was decreased to 13 °C, whereas no binding of Yb[±] was detected at 37 °C (see Fig. 7) because of the partial unfolding of Cla h 8 at this temperature. In addition, the ability of Cla h 8 to discriminate between ssDNA and dsDNA was found to be higher at 25 °C than at 13 °C, as becomes obvious from comparing the dissociation constants of Yb[−] and Yb[±] at these two temperatures (see Table I).

DISCUSSION

Cla h 8 was identified as an IgE-binding protein from a *C. herbarum* phage surface-displayed cDNA library. This minor allergen of *C. herbarum* exhibited high sequence homology with bacterial CSPs and was therefore identified as the first eukaryotic cold shock protein that is not a functional domain

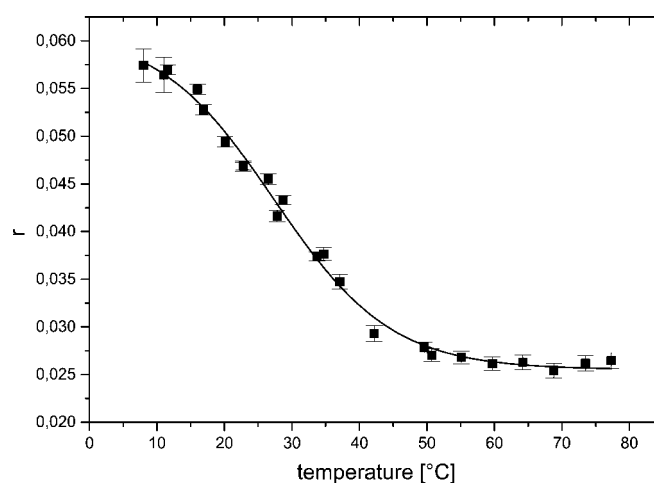


FIG. 6. Temperature dependence of the fluorescence anisotropy of 300 nM Cla h 8. The values shown represent the mean values of three independent measurements. The transition point was calculated by fitting the data to the Boltzmann equation.

TABLE I
Dissociation constants of Cla h 8

The dissociation constants of the interaction of Cla h 8 with different ligands were obtained by analysing the fluorescence binding isotherms according to Eq. 1.

Temperature	Interaction	K_d
°C		$\times 10^{-9}$ M
13	Cla h 8/Yb [−]	4.7 ± 1
	Cla h 8/Yb [−]	5.9 ± 1.8
	Cla h 8/Yb [±]	7.6 ± 1
	Cla h 8/Yb ⁺	11 ± 1.7
25	Cla h 8/Yb [−]	3.2 ± 1.3
	Cla h 8/Yb [−]	3.2 ± 1.3
	Cla h 8/Yb [±]	27.4 ± 3.1

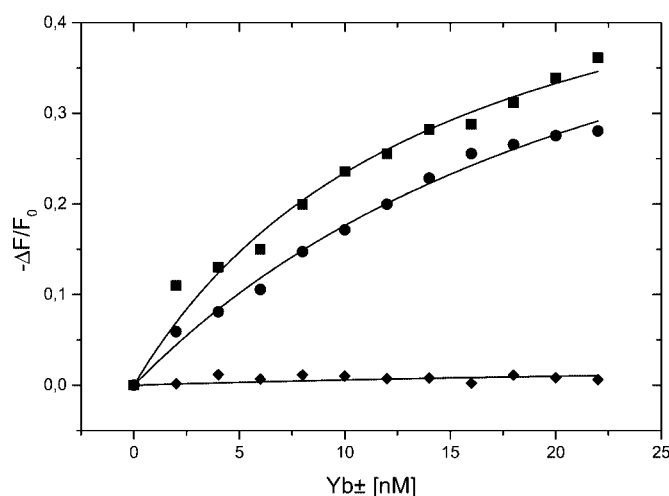


FIG. 7. Fluorescence binding isotherms of Cla h 8 interacting with Yb[±] at 13 (■), 25 (●), and at 37 °C (◆). The normalized mean changes in fluorescence intensity ($-\Delta F/F_0$) resulting from three independent experiments are plotted against the ligand concentration. The resulting binding isotherms were fitted by non-linear regression to Eq. 1 to obtain the dissociation constants, K_d as described previously (17, 18, 45, 46).

of a Y-box binding protein. In prokaryotes, little is known about the involvement of CSPs in dsDNA processing. Although CspA was shown to act as a cold shock transcriptional enhancer of at least two genes in *E. coli*, the protein lacks *in vitro* any ability of binding to dsDNA (49, 50). In eukaryotes, Y-box binding proteins act as transcription factors, and they were shown to bind to dsDNA *in vitro*. The cold shock domain

of Y-box binding proteins was found to be necessary but not sufficient for the interaction with dsDNA (27). Recently, the basic amino acids-containing loop between the $\beta 3$ and $\beta 4$ strands of the human Y-box binding protein 1 was shown to be important for dsDNA binding, and that this loop, when incorporated into CspA, induced dsDNA binding in this bacterial CSP (26). However, this loop was not found in Cla h 8, although the protein's ability to bind to dsDNA, especially at low temperatures, is striking (see Fig. 7). It can therefore be hypothesized that Cla h 8 represents an evolutionary link between prokaryotic CSPs and eukaryotic cold shock domains contained within Y-box-binding proteins.

Structurally, the overall fold of Cla h 8 is assumed to resemble the all- β -sheet motif of bacterial CSPs. This is due to the high sequence homology of Cla h 8 with bacterial CSPs, which allowed the modeling of a reliable three-dimensional structure of the allergen. The high β -sheet content of the protein became manifest in the far-UV CD spectra of the protein (see Fig. 3). However, Cla h 8 seems to be much less stable than bacterial CSPs, exhibiting thermal unfolding transitions at 35.7 or at 28 °C (see Figs. 4 and 6) depending upon the method used; unfolding monitored by CD spectroscopy refers to a transition of the protein's secondary structure, whereas fluorescence anisotropy refers to a transition of the protein's tertiary and quaternary structure. The different transition temperatures found by the two methods, which reflect different structural features, refer to a multistate thermal transition of Cla h 8 (51).

The low unfolding transition of Cla h 8 might refer to a short lifetime of the uncomplexed protein *in vivo*. Thermal stability of Cla h 8 was found to increase by the addition of ssDNA (see Fig. 5). We have recently shown that in the case of nucleotide or carbohydrate binding the thermal stability of proteins like transcription factors or chemokines can be improved significantly (52, 53). Additional intramolecular bonds between the receptor and the ligand lead, if exothermic, together with a reduction of the degrees of freedom to a significant stabilization of the protein structure. Cla h 8 can therefore be postulated to bind with high affinity to nucleic acids *in vivo* to increase its own stability. If thereby the stability of, for example, mRNA becomes decreased Cla h 8 acts as a so-called RNA chaperone, a function which has long been proposed for CSPs (21).

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