Aromatic Amino Acids Are Critical for Stability of the Bicoid Homeodomain*

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The Drosophila Bicoid (Bcd) protein plays a dual role as a transcription and translation factor dependent on the unique DNA and RNA binding properties of the homeodomain (HD). We have used circular dichroism and fluorescence spectroscopy to probe the structure and stability of the Bcd-HD, for which a high resolution structure is not yet available. The fluorescence from the single tryptophan residue in the HD (Trp-48) is strongly quenched in the native state but is dramatically enhanced (~20-fold) upon denaturation. Similar results were obtained with the Ultrabithorax HD (Ubx-HD), suggesting that the unusual tryptophan fluorescence may be a general phenomenon of HD proteins. We have used site-directed mutagenesis to explore the role of aromatic acids in the structure of the Bcd-HD and to evaluate the proposal that interactions between the strictly conserved Trp residue in HDs and nearby aromatic residues are responsible for the fluorescence quenching in the native state. We determined that both Trp-48 and Phe-8 in the N-terminal region of the HD are individually necessary for structural stability of the Bcd-HD, the latter most likely as a factor coordinating the orientation of the N-terminal helix I and the recognition helix for efficient binding to a DNA target.

Because of this unique DNA and RNA binding capability, Bcd-HD is a particularly compelling target for investigations of molecular structure and nucleic acid recognition mechanisms.

The HD has also become a paradigm for studying DNA-protein interactions. HDs fold into a module with a characteristic structure comprising three α-helices. Structural studies, first elucidated for the Antennapedia and Engrailed HDs, show that the three helices of the HD are stabilized by hydrophobic interactions involving residues Leu-16, Trp-48, and Phe-49, which are conserved in all HDs (9–15). The HD shows a common helix-turn-helix motif encompassing the recognition helix III, which docks into the major groove of DNA. The specificity of DNA recognition is achieved by specific amino acid residue-base contacts in helix III (16), e.g. amino acid residue 50 (glutamine in most HDs, lysine in the case of Bcd-HD) both in vitro and in vivo (4, 7, 17–21). Despite the extensive data on sequence and structure-function relationships accumulated over the past years, some aspects of HD function still remain obscure. Little is known about how HDs achieve specific recognition of their targets, the role of the N-terminal arm, and the effects of other highly conserved residues, mostly within helix III. Among these, the centrally located Trp residue (Trp-48) is invariant. The available structural data indicate that Trp-48 plays a role in DNA-protein interactions through a water-mediated hydrogen bond with the phosphate backbone (10–14). However, the absolute conservation of Trp-48 in evolution indicates a far more important function in the HD.

We have used biophysical methods combined with site-directed mutagenesis to elucidate the molecular structure-function relationships of the Bcd-HD, for which a high resolution structure is not yet available and have focused on the highly conserved aromatic residues tryptophan (Trp-48, helix III), and phenylalanine (Phe-8, helix I). In homeodomains, Trp-48 exhibits a strongly quenched fluorescence in the native state (22–24) most likely because of specific interactions with neighboring residues. Conservative mutation of the strictly conserved Trp-48 to a phenylalanine (W48F) reduces DNA binding affinity and destabilizes the HD, suggesting that the role of Trp-48 is to stabilize critical structural features required for DNA recognition. Replacement of Phe-8 by a tyrosine residue (F8Y, Antennapedia like) yields a stable HD with fluorescence and binding properties similar to the wild-type HD. Replacement of Phe-8 by an alanine (F8A) yields a less stable HD, with lower quenching of Trp-48 fluorescence in the native state. These results suggest the following. (i) An aromatic residue in position 8 may be required to provide the scaffold, holding the HD in a conformation required for DNA binding and (ii) that the quenching of Trp-48 in the native state has more complex origins.

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The abbreviations used are: HD, homeodomain; Bcd, Bicoid; Ubx, Ultrabithorax; GdmHCl, guanidinium hydrochloride.
MATERIALS AND METHODS

Protein Expression and Purification—The recombinant protein (Bcd-HD) was expressed and purified as described (8). Protein purity was confirmed by SDS-polyacrylamide gel electrophoresis. Further purification was performed as required over an Amersham Pharmacia Biotech mono-P column in 10 mM triethanolamine-HCl buffer, pH 7.0. Site-directed mutagenesis of Bcd-HD was carried out using the Stratagen QuickChange mutagenesis kit using the following primers and the wild-type Bcd-HD plasmid pRSETBcdHDCHis (8) as a template: W48F, 5'-GCCCAAGTGGAAGATTTTTTAAAGACCGTCGCCTC-3' and 5'-GACGCCGACGGTCTTTAAAAATATCTTATCCTGGCCG-3', F5Y, 5'-CGTCGCCACCCGCACCACTGCTACCAGCTCTCAAATAG-3'.

Secondary structure prediction was performed as described (8). A secondary structure prediction algorithm applied to the spectra yielded an α-helical content of 41% (Fig. 1A), and similar to that observed for the native Ubx-HD, and F8A, 5'-CGTCGCCACCCGCACCACTGCTACCAGCTCTCAAATAG-3', 5'-CTATTTGAGAGCTGGTGACGTAGTGGTGCGGGTGCGACG-3' and 5'-CTATTTGAGAGCTGGTGACGTAGTGGTGCGGGTGCGACG-3'.

Steady-State Fluorescence—Fluorescence was measured on an SLM 8000C (SLM, Urbana, IL) or a PTI Quantamaster (Photon Technology International, Lawrenceville, NJ) photon counting spectrophotofluorometer with a 10-nm bandpass filter, and the excitation at 280 nm was through a 10-nm bandpass filter, and the emission was selected with a 325-nm long-pass filter. The reference spectra of the Bcd-HD under native conditions showed a double minimum at 208 and 222 nm, characteristic of a helical structure (Fig. 1B). A fit to the thermal denaturation data yielded a Tm = 44 °C.

CD Spectroscopy—CD measurements were performed on a JASCO J-720 CD spectropolarimeter. Far-UV CD data are presented as molecular ellipticity [θ] in deg cm² dm⁻¹ mol⁻¹. Secondary structure was predicted using the SSE-338 prediction algorithm as described by the Trp-48 fluorescence produced a similar Tm.

CD Spectra of the Wild-Type Bcd-HD and the Bcd-HD W48F Mutant. a, equilibrium CD spectra of wild-type Bcd-HD (solid line) and Bcd-HD W48F mutant (solid symbols). Inset, secondary structure estimation parameters using the algorithm of Yang et al. (25) for both Bcd-HD and the Bcd-HD W48F mutant. b, thermal denaturation of Bcd-HD wild type monitored by [θ]₂₂₂. Fits to the pre- and post-transition baselines are shown. T_m = 44 °C.

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In Vitro Binding Assays—The Bcd target site oligonucleotides (5'-AATCTTTACCTCAATA-3') were 5'-F³₂-P-labeled with polynucleotide kinase (29). Gel shift assays were carried out as described (7). Briefly, DNA (10 fmol) and protein were mixed in 25 mM potassium chloride, 7.5, 0.1 M KCl, 12.5 mM MgCl₂, 0.1% Nonidet P-40, 20% glycerol at 4 °C for 15 min. Free and complexed DNA were resolved by electrophoresis on 12% polyacrylamide gels in 0.5× TBE buffer at 12 V/cm. The DNA concentrations were sufficiently low so that the fraction of bound protein was negligible. Quantitation was performed with a PhosphorImager (Molecular Dynamics, Sunnyvale, CA).

RESULTS

The Bcd-HD Has Substantial Helical Structure—The CD spectra of the Bcd-HD under native conditions showed a double minimum at 208 and 222 nm, characteristic of a helical structure (Fig. 1A), and similar to that observed for other homeodomains (21, 30, 31). A secondary structure prediction algorithm applied to the spectra yielded an α-helical content of 41% (Fig. 1a, inset). As noted by Ades and Sauer for the Engrailed HD (21), the CD signal at 222 nm was somewhat lower than that expected for a protein with a ~60% helical content, but the discrepancy can be attributed to partial fold and decreased aromatic residues in the sequence (32-33). We monitored the loss of helical structure upon thermal denaturation of the Bcd-HD by measuring the CD signal at 222 nm as a function of temperature (Fig. 1b). The protein exhibited a two-state folding transition with a T_m = -44 °C, comparable with the value reported for the rat TTF-1 HD (42.8 °C) (31) and the Antennapedia HD (48 °C) (34). A fit to the thermal denaturation data yielded a ΔH_m = -38 kcal mol⁻¹. The unfolding of the Bcd-HD monitored by the Trp-48 fluorescence produced a similar T_m.

Trp Fluorescence Is Strongly Quenched in the Native State of the Bcd-HD and Ubx-HD—We further explored the structure of the Bcd-HD using the fluorescence of Trp-48. Trp-48 was excited selectively at 295 nm to avoid contributions from the single Tyr residue in the sequence, Tyr-25. In the folded state, the emission was strongly quenched (95%) relative to that in the unfolded state (Fig. 2a). The protein fluorescence excited at 280 nm was dominated by Tyr-25 and had an emission peak at 314 nm (Fig. 2b). Upon unfolding in 6 M guanidinium hydrochloride (GdmHCl), the intensity of the Trp-48 fluorescence (excited at 295 nm) increased by a factor of ~20 and was accompanied by a 15-nm red-shift in the emission spectra (Fig. 2c). A similar result was obtained for unfolding in urea (data not shown). For comparison, we studied the fluorescence properties of the Ubx-HD. This HD also contains a single Trp (Trp-48) but unlike Bcd-HD has three Tyr residues (Tyr-8, Tyr-11, and Tyr-25). As in the Bcd-HD, the Trp-48 fluorescence of the Ubx-HD in the native state was strongly quenched (Fig. 2d). This result suggests that the quenching of Trp-48 fluorescence is not restricted to the Bcd-HD but may rather be a property of homeodomains in general, implying that Trp-48 may serve as a sensitive conformational marker for studying the overall conformation of homeodomain protein family. Similar results have been observed for other homeodomains by Nanda and Brand (23).

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both native and denatured lifetimes were multi-exponential and that the analysis of the native state fluorescence lifetime was difficult because of the potential contamination by even a minute fraction of denatured or partially denatured polypeptide, leading to a perturbation of the mean lifetime to longer values. Nonetheless, the trend (reduction of fluorescence lifetime, i.e. quantum yield, in the native state) was clear in both cases.

**Mutation of Trp-48 Abolishes DNA Binding**—We mutated Trp-48 in the recognition helix to a Phe to evaluate the role of this residue in determining the structure and binding properties of the Bcd-HD. Phe was selected as a conservative replacement for Trp-48 to avoid disrupting the immediate environment of helix III. The equilibrium CD spectra of Bcd-HD W48F revealed the characteristic helical minima at 208 and 222 nm (Fig. 1a), with a predicted helical content of ~35% (Fig. 1a, inset), suggesting that the mutation did not have significant effects at the level of secondary structure. However, electrophoretic mobility shift assays showed that Bcd-HD W48F bound DNA with significantly reduced affinity compared with the wild-type Bcd-HD (Fig. 3; a, b and e). This result was not consistent with the limited effect on secondary structure. Furthermore, structural data based on x-ray crystallography of HDs had indicated that Trp-48 is not critically involved in DNA binding, providing only indirect contacts to the phosphate backbone. It follows that the pronounced reduction of affinity for DNA could not be attributed solely to a slight change in secondary structure or loss of a weak contact to DNA.

To further investigate the origin of the impaired DNA binding of Bcd-HD W48F, we analyzed the stability of the mutated HD. The thermal denaturation of Bcd-HD W48F monitored by CD spectroscopy revealed a sharp reduction in the Tm from 44 to 27 °C (Fig. 4), suggesting that the mutation had a strong destabilizing effect. We conclude that Trp-48 imparts rigidity to the helix-turn-helix motif of the homeodomain, perhaps thereby potentiating DNA binding.

As expected, the Bcd-HD W48F exhibited a fluorescence spectrum characteristic of the single Tyr-25 in the mutant protein. Upon unfolding Bcd-HD W48F in GdmHCl or urea, the fluorescence intensity increased by ~50% (data not shown).

**An Aromatic Residue in Position 8 Is Required for Stability of the HD**—We mutated the native phenylalanine residue in position 8 of the HD to an alanine or to a tyrosine (similar to that of the Antennapedia HD, which is often described as the canonical HD). Both mutants exhibited significant helical structure measured by CD (data not shown). From the electrophoretic mobility shift assay, Bcd-HD F8A had a similarly reduced affinity to DNA as Bcd-HD W48F, whereas the Antennapedia-like Bcd-HD F8Y mutant retained the wild-type binding affinity (Fig. 3, c–e). The Bcd-HD F8A mutant was destabilized (Tm 46 °C) than the wild-type HD (Tm 37 °C) whereas the Bcd-HD F8Y mutant is slightly more stable (Tm 46 °C) than the wild-type HD (Tm 44 °C, see also Fig. 4).

Fluorescence spectra of native and denatured Bcd-HD F8A and Bcd-HD F8Y mutants are shown in Fig. 2, e and f. The Trp fluorescence intensity of denatured Bcd-HD F8A was ~11 times that of the native Bcd-HD F8A, whereas denatured Bcd-HD F8Y showed an intensity ~23 times larger than the native Bcd-HD F8Y. Clearly there was a correlation between the presence of an aromatic residue in position 8 of the HD and the quenching of native Trp fluorescence, but this interaction could not account for the full extent of the quenching.

**Molecular Modeling of Bcd-HD Structure**—Knowledge-based protein modeling methods (SWISS-MODEL automated protein modeling server) were used to generate a model for the structure of the Bcd-HD (Fig. 5, a and b) based on the coordinates of existing homeodomain structures. The relative orientations of the three helices and the positions of Trp-48 and other aromatic residues (Phe-8, Phe-20, Tyr-25, Phe-49) in the Bcd-HD are shown in this model.

**DISCUSSION**

We have probed the structural characteristics of Bcd-HD, a nucleic acid binding domain specifically recognizing RNA and
DNA in its capacity as a dual regulator of gene expression and translation, by exploiting the single Trp in the molecule (Trp-48), a strictly invariant residue in the primary sequences of the HD family, as a sensitive probe of conformational changes in the protein. The strict conservation of Trp-48 in the HD family suggests that it plays a key role in the function of homeodomains. Although Trp-48 lies in the recognition helix, it is not involved in direct contacts with the DNA but rather makes a water-mediated hydrogen bond with the phosphate backbone (12, 15, 37).

Structural Aspects—The CD spectra confirmed that the α-helical content of the Bcd-HD is similar to that of other HDs.

From the thermal denaturation profile, the Bcd-HD is an autonomously folded domain exhibiting a reversible two-state unfolding transition. The Tm (44 °C) is comparable with that of other known homeodomains (30, 31). The conservative mutation W48F, designed to minimally perturb the overall hydrophobicity of the helix III environment, has a significant α-helical structure yet exhibits 50-fold lower affinity for DNA, suggesting that Trp-48 plays a more complex role in DNA binding than that postulated previously. In addition, the dramatic destabilization (reduction of Tm by ~17°) of the mutant confirms that Trp-48 forms an integral part of the hydrophobic core of the HD. Trp-48 and Phe-49 are conserved in all known HDs and must play a role in stabilizing the folded structure and determining the spatial relationships required for DNA recognition (12). In addition to a direct role in DNA binding, a major function of Trp-48 in the helix III of the HD is to stabilize the global structure, thus allowing other residues to make critical DNA contacts.

Trp-48 Fluorescence Properties—According to the relatively blue-shifted fluorescence peak (329 nm) in the native state, Trp-48 is buried within the molecule and thus shielded from solvent. The few known HD structures and molecular modeling of the Bcd-HD are consistent with these data. The fluorescence of Trp-48 increased dramatically (20-fold) upon denaturation. That is, Trp-48 fluorescence displays an unprecedented degree of quenching in the native state. Ubx-HD also possesses a single Trp residue that is highly quenched (this work and Ref. 23). In view of the observations of Nanda and Brand (23, 24) of quenching in the native state, the fluorescence properties of Trp-48 may be general in the context of the HD fold and thus applicable to all HDs. In other words, Trp-48 fluorescence may be useful as a probe for comparing the structures of other members of this family for which high resolution structural information is not yet available.

Role of Phe-8—A surprising result of this work is the importance of the aromatic residue in position 8. The Bcd-HD F8A mutant shows a significantly reduced DNA binding ability (comparable with W48F) and a destabilized structure. Conversely, the Bcd-HD F8Y mutant binds as well as the wild-type HD and is slightly more stable. This suggests that an aromatic residue in position 8, and the resultant interaction with Trp-48, may be critical to mediate the structure of the HD by bringing helix I and the recognition helix, helix III, into a conformation optimal for DNA binding.

Mechanisms of Trp-48 Fluorescence Quenching—Carra and Privalov (30) reported quenching of fluorescence in the native
state of the MATO2-HD and suggested that interactions with adjacent residues might be responsible. Native state quenching was also observed in the trp aporepressor protein (38) and recently in the immunophilin domain of the FK506-binding protein FKBP59-I (39). FKBP59-I contains two Trp residues, one of which is deeply buried in the hydrophobic core and which fluoresces very weakly compared with the second, surface-exposed, Trp residue. The quenching of this residue was attributed to an atypical hydrogen-bond interaction involving the indole nitrogen of the buried Trp and the benzene ring of a neighboring Phe residue. In this conformation, the indole NH group points toward the center of the Phe benzene ring, which can act as a hydrogen-bond acceptor interacting with a donor (40). The strength of this interaction (−3 kcal mol−1) is about half that of commonly occurring H-bonds (40) and 5×kT at room temperature. Thus, it could have a significant role in stabilizing molecular interactions. Burley and Petsko (41) found that such interactions between amino and aromatic groups occur more frequently than expected. In FKBP59-I, the planes of the aromatic rings of the interacting Trp and Phe residues are oriented almost perpendicular to each other, which could be expected to facilitate the quenching more than a parallel configuration. Using time-correlated single photon counting techniques, Suwaiyan and Klein (42) measured a dramatic decrease in the lifetime of indole in cyclohexane solution upon the addition of benzene and suggested that the quenching was because of H-bonding between the indole NH and the π system of benzene. In addition, Van Duuren (43) showed that the fluorescence intensities of 1,2-dimethylindole and 1-methyl-2-phenylindole do not decrease in benzene solution relative to other solvents, implying that the free indole NH is essential for such complex formation. These results correlate well with the observed decrease of the lifetime of Trp-48 fluorescence in Bcd-HD and Ubx-HD. Nanda and Brand (23) have used similar arguments to suggest that the Trp-Phe NH−π hydrogen bond is responsible for the quenching of the Trp fluorescence in HDs and have proposed an excited state rearrangement model in which a simple rotation of the Trp around the Cα-Cβ bond would be sufficient to bring the indole nitrogen in proximity to the aromatic acceptor on the N-terminal arm. In the absence of a x-ray crystallographic or solution NMR-derived structure for the Bcd-HD, one cannot state conclusively that the proposed interaction plays a role in the quenching of the Trp-48 in the Bcd-HD. Closer inspection of the surroundings of Trp-48 in the homologous-based molecular model of the Bcd-HD reveal a possible interaction of the indole nitrogen with the benzene ring of Phe-8 (Fig. 5b; ~4 Å distance of closest approach), which might contribute to the quenching of Trp-48. In fact the planes of the aromatic rings of the two residues are almost orthogonal to each other (Fig. 5b). Similarly, although a high-resolution structure of the Ubx-HD is not available, it is plausible that an interaction between Trp-48 and the aromatic ring of Tyr-8 in Ubx-HD also contributes to the fluorescence quenching. A survey of the known homeodomains reveals that all, except for the LIM and POU class proteins, possess a Tyr or Phe residue at position 8. However, as our fluorescence data for the Bcd-HD F8A and Bcd-HD F8Y mutants (Fig. 2, e–f) indicate, this interaction alone cannot account for the full extent of the quenching. That is, other mechanisms or interactions must intervene, possibly involving other neighboring aromatic residues, which are clustered around Trp-48 (Fig. 5b). A likely candidate is Phe-49, which is also nearly invariant in HDs with the exception of the MATa2 (valine) and DmBarHI and DaBarHI (tyrosine) (44).

Chen and Barkley (45) have recently identified amino acid side chains that quench Trp fluorescence in solution. Lys and Tyr residues quench by excited-state proton transfer whereas Gln, Asn, Glu, Asp, Cys, and His quench by electron transfer. Electron transfer has less stringent geometric requirements than proton transfer, can occur through-space or through-bond over distances of up to 10 Å, and is thus likely to play a major role in quenching. In the Bcd model structure described above, His-19 and His-56 are within 10 Å of Trp-48. However the ionization state of His residues critically affects their quenching ability, with positively charged His residues being much more effective than neutral His residues. We would not expect His-19 or His-56 to be protonated under our experimental conditions. The quenching ability of other amino acid side-chains such as Arg, of which a stretch of four residues occurs in helix III of the Bcd HD, might be enhanced by the proximity and orientation of these side-chains in the context of the protein fold, even though at neutral pH these show negligible quenching of model compound fluorescence (45).

Further insight into the dynamics and nature of the interactions of the indole proton of Trp-48 of Bcd-HD with surrounding residues will be provided by hydrogen/deuterium exchange experiments and by progress in the structure determination of the Bcd-HD.

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REFERENCES

Characterization of the Bicoid Homeodomain