Journal of Biochemistry and Molecular Biology, Vol. 34, No. 1, January 2001, pp. 1-7

JOURNAL OF
Biochemistry and Molecular Biology

© BSRK & Springer-Verlag 2001

The Homeobox and Genetic Disease: Structure and Dynamics of Wild Type and Mutant Homeodomain Proteins

James A. Ferretti*
Laboratory of Biophysical Chemistry National Heart, Lung & Blood Institute
National Institutes of Health Bethesda, MD 20892-0380 USA

Received 3 November 2000

Structural and physical properties of type wild type and various selected mutants of the vnd/NK-2 homeodomain, the protein product of the homeobox, and the implication in genetic disease are reviewed. The structure, dynamics and thermodynamics have been investigated by NMR and by calorimetry. The interactions responsible for the nucleotide sequence-specific binding of the homeodomain to its consensus DNA binding site have been identified. There is a strong correlation between significant structural alterations within the homeodomain or its DNA complex and the appearance of genetic disease. Mutations in positions known to be important in genetic disease have been examined carefully. For example, mutation of position 52 of vnd/NK-2 results in a significant structural modification and mutation of position 54 alters the DNA binding specificity and affinity. The ^{15}N relaxation behavior and heteronuclear Overhauser effect data was used to characterize and describe the protein backbone dynamics. These studies were carried out on the wild type and the double mutant proteins both in the free and in the DNA bound states. Finally, the thermodynamic properties associated with DNA binding are described for the vnd/NK-2 homeodomain. These thermodynamic measurements reinforce the hypothesis that water structure around a protein and around DNA significantly contribute to the protein-DNA binding behavior. The results, taken together, demonstrate that structure and dynamic studies of proteins combined with thermodynamic measurements provide a significantly more complete picture of the solution behavior than the individual studies.

Keywords: Homeobox, Homeodomain, vnd/NK-2

*To whom correspondence should be addressed.
Fax: 1-301-402-3405
E-mail: jafer@helix.nih.gov

Introduction

The primary purpose of this manuscript is to review several aspects of structural and biochemical information on the homeodomain and the corresponding relation to genetic disease. Many researchers contributed to the studies described herein and they are sited in the Acknowledgements. The basis of this review arises from a plenary lecture given at the 2000 Fall Scientific Meeting of the Biochemical Society of the Republic of Korea. In that lecture I described the likely relationship between the phenotypic manifestation of a genetic disease and alterations in the three-dimensional structure of the related protein or in the nature of the binding of the protein to DNA. The lecture was limited to discussion of genetic disease or altered embryonic development that are correlated with single amino acid residue mutations in the homeodomain. In this paper my motivation is to further describe structural alterations in the homeodomain or the homeodomain-DNA complex that are directly related to genetic disease in the case of humans or to altered embryonic development for lower species.

The homeodomain is the highly conserved DNA-binding domain of a class of proteins that regulate transcription in the commitment of embryonic cells to specific developmental pathways (Scott *et al.*, 1989; McGinnis & Krumlauf, 1992; Gehring *et al.*, 1994). The homeodomain part of the protein is encoded by the homeobox, which is found in all eukaryotic species from plants and yeast through humans. The homeobox gene is often expressed at very early stages in embryonic development and is responsible for the regulation of other downstream target genes.

Homeodomains are protein segments of usually 60 amino acid residues in length. They function by recognizing and binding specific nucleotide sequences in DNA and are responsible for the DNA binding of the entire protein encoded by the gene that contains the homeobox. Knowledge of the nature and specificity of the homeodomain-DNA contacts is important to the understanding of developmental regulation.

The ability of the homeodomain to function properly is dependent upon its capacity to adopt the correct three-dimensional structure and to properly bind a specific sequence or set of sequences of DNA (Gruschus *et al.*, 1997). Members of the homeodomain family show a high degree of homology in their tertiary structures, possessing three helical segments including a helix-turn-helix DNA binding motif. The N-terminal arm and helix III contact the DNA and make specific interactions with the bases and phosphate backbone. This capability to bind properly to DNA and function relies on various factors that include solution conditions, sequence, structure and motional properties.

Conservation of residues in any given homeodomain appears to be critical for proper function. Single amino acid residue replacements; i.e., mutations, in the homeodomain result in or cause altered embryonic development (Jiminez *et al.*, 1995) or genetic disease (Schott *et al.*, 1998). Numerous examples are now available of these single amino acid residue replacements being directly associated with genetic disease in humans or altered embryonic development in lower species. A particularly dramatic example has been demonstrated for a cardiac specific homeodomain, CSX/NKX-2.5 (Kasahara *et al.*, 2000). The NKX-2.5 homeodomain is a member of the NK-2 class of homeodomains from *Drosophila melanogaster* first described by Kim and Nirenberg (1989). This CSX/NKX-2.5 homeodomain is an evolutionarily conserved homeodomain containing transcription factor that is essential for early cardiac development in humans. Recently, different heterozygous mutations were found in patients with congenital heart defects that are transmitted in an autosomal dominant fashion. Four of the mutations involved single amino acid residue replacements within the homeodomain (Fig. 1) and lead to such anomalies as atrial septum disease, ventricular septum defect, tetralogy of Fallot, and tricuspid valve abnormality (Benson *et al.*, 1999).

Single residue replacements can result in structural and dynamic changes in the homeodomain, a modification of the physical properties such as the thermal unfolding or 'melting' temperature, or an alteration in the affinity and target specificity for the DNA. The ability to relate anomalies in function to such structural or dynamical alterations in a transcriptional protein provides one of the simplest and most direct opportunities to investigate the structure-function relationship.

MUTATIONS IN NKX-2.5 RELATED TO CONGENITAL HEART DISEASE

Thr 41	⇨	Met
Asn 51	⇨	Lys
Arg52	⇨	Gly
Tyr 54	⇨	Cys

Fig. 1. Single amino acid mutations found in the cardiac specific homeodomain NKX-2.5 that result in congenital heart disease.

Materials and Methods

The procedures and protocols for the expression and purification of the wild type and mutant vnd/NK-2 homeodomains has been described elsewhere (Weiler *et al.*, 1998; Xiang, *et al.*, 1998). The proteins are expressed from the pET15b rector in Esterichia coli grown either in minimal media or singly ^{15}N labeled or double $^{15}N/^{13}C$ labeled media. Purification of the protein product is typically carried out by passing the expression mixture over an affinity purification column and then eluting the product with increasing concentrations of histidine.

All NMR spectra were obtained on Bruker AMX360, DRX600, or DRX800 NMR spectrometers. Details of the pulse sequences employed and the specific procedures used are described in the relevant publications.

Internuclear distance restraints were obtained for the structure determinations from the appropriate NMR experiments. Dihedral angle restraints were also employed to improve the quality of the structure determinations. Hydrogen bond restraints were added for slowly exchanging backbone amide protons whose hydrogen bonded partners were clearly evident. Ensembles of structures typically were generated using InsightII (Molecular Simulations, Inc., San Diego, CA). The details of the protocol employed for each structure determination are described elsewhere (Gruschus *et al.*, 1999).

Summary of Results

To investigate the structure and dynamics of the homeodomain and subsequently the alterations induced by single amino acid residue mutations, the parent homeodomain from the NK-2 class, vnd/NK-2. The NK-2 class of homeodomains is defined by the presence of tyrosine in position 54. The sequence of the vnd/NK-2 is given together with that of the antennapedia homeodomain and the cardiac specific homeodomain, NKX-2.5.

```
vnd/NK-2 K R K R R V L F T K A Q T Y E L E R R F
NKX-2.5  R R K P R V L F S Q A Q V Y E L E R R F
ANTP     R K R G R Q T Y T R Y Q T L E L E K E F

                             30                40
vnd/NK-2 R Q Q R Y L S A P E R E H L A S L I R L
NKX-2.5  K Q Q R Y L S A P E R D Q L A S V L K L
ANTP     H F N R Y L T R R R R I E L A H A L C L

                             50                60
vnd/NK-2 T P T Q V K I W F Q N H R Y K T K R A Q
NKX-2.5  T S T Q V K I W F Q N R R Y K C K R Q R
ANTP     T E R Q V K I W F Q N R R M K W K K E N
```

The antennapedia homeodomain is shown, since it was the first homeodomain to be identified and the first homeodomain

Fig. 2. Comparison of the secondary structures of the wild type and H52RT56W double mutant vnd/NK-2 homeodomain.

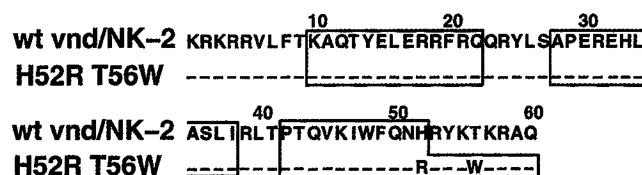

Fig. 3. Superposition of the protein backbone of the 30 structures of the vnd/NK-2 homeodomain. The colors are representative of the r.m.s.d. of the 30 structures, where blue indicates very well-defined, gree/yellow indicate intermediate and magenta indictes not well-defined.

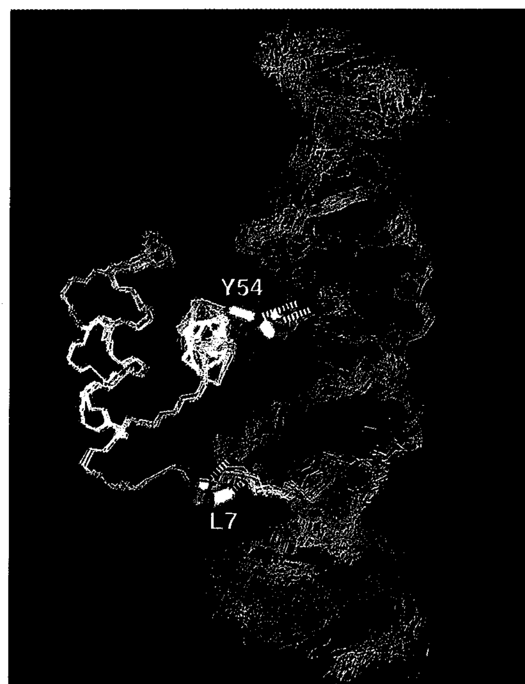

Fig. 4. Superposition of the ensemble of 20 NMR structures of the vnd/NK-2 DNA complex, superposing simultaneously the well-ordered protein backbone and well-ordered DNA base-pairs. Residues 7 and 54 are indicated in detail to highlight their interactions with the DNA. The N-terminal arm of the homeodomain is inserted into the minor groove of the DNA, and helix III, the DNA recognition helix, is inserted into the major groove. The coloring indicates the atomic r.m.s.d from the average structure.

structure reported. The vnd/NK-2 gene is a neural gene regulator that initiates the neural program of development in part of the central nervous system of *Drosophila* embryos. The vnd/NK-2 homeodomain binds ($K_d = 0.2$ nM) to an unusual DNA consequence that contains 5'-CAAGTG-3' as its core. The three-dimensional structure was obtained by NMR spectroscopy with two protein samples that are uniformly singly enriched in ^{15}N and uniformly double enriched in ^{15}N and ^{13}C. The sample uniformly enriched in ^{15}N provides primarily information on the backbone and secondary structure of the protein. The sample uniformly double enriched in ^{15}N and ^{13}C provides additional backbone information as well as resonance assignment and structural distance on the sidechains. The structure of the unbound or free homeodomain was determined by NMR spectroscopy in solution because the vnd/NK-2 homeodomain does not crystallize in the absence of DNA. The solution structure actually was performed on a 77 amino acid residue fragment that encompasses the homeodomain (Tsao *et al.*, 1995). The structure was determined using a total of 986 internuclear proton distance restraints and 55 backbone ϕ angle restraints. With these distance and angle restraints the structures of the homeodomain initial were generated using the program DSPACE. Further refinement was carried out with the program XPLOR employing a parameter set where all energy terms were included except the electrostatic term. The refinement cycles consisted initially of energy minimization, 5 ps of dynamics at 600 K with no gradual heating and the cooling to 300 K. The homeodomain contains three helical segments and forms a helix-loop-helix-turn helix structure (Fig. 2). Helix I extends from residues 10 to 22, helix II from residues 28 to 38, a turn, from residues 39 to 41 and helix III, from residues 42 to 52. A superposition of the final 30 structures obtained is shown in Fig. 3. An interesting result of this structure determination is that helix III, the DNA recognition helix, comprises only 10 helix residues, which is shorter that that normally found for the homeodomains. In addition, the secondary structures, DNA binding properties, and thermal denaturation behavior of several site-directed mutants of the homeodomain encoded by the *vnd/NK-2* gene have been reported. Mutations of variable residues in two positions in the vnd/NK-2 homeodomain, namely H52R and T56W result in a significant increase in the length of helix III in the absence of DNA.

Subsequently, the three-dimensional solution structure obtained by NMR of the complex formed between the homeodomain and its consensus 16 base-pair DNA binding

sequence was determined (Gruschus *et al.*, 1999). The conformation of the vnd/NK-2 homeodomain bound to DNA is typical of the homeodomains. A superposition of 20 structures of the homeodomain-DNA complex is shown (Fig. 4) with tyrosine-54 (Y54) and leucine-7 (L7) specifically depicted. Helix III is inserted into the major groove of the DNA and makes the majority of specific intermolecular contacts with the DNA bases. Helix III lengthens at its C-terminal end upon binding to DNA, going from 10 to 20 residues (i.e. from residues 42 to 60). Both helix I and helix III are 'N-capped', where the backbone amide proton of the third residue I of each of the respective helices, which is normally exposed to solvent, is hydrogen bonded to the side-chain oxygen atom of threonine occurring in the respective I-3 positions, T9 and T41. Although helix II could be 'N-capped', experimental data provide no evidence to support N-capping for vnd/NK-2 here.

The conclusions concerning atom-atom interactions that result from the determination of the structure of the vnd/NK-2 homeodomain bound to DNA provide a detailed understanding of the role of individual amino acid residues. While there is a high degree of tertiary structural homology across the homeodomains, variations due to individual amino acid residues are important. This global homology eventually will allow establishment of rules governing structural, DNA binding behavior and biological function of the various amino acid residues in each of the 60 positions of the homeodomain. It seems apparent that the ability of the homeodomain to function properly is a delicate and finely tuned device and that even the smallest of changes can result in significant functional consequences.

The contact between Y54 and the (-)-C5 of the DNA consensus sequence gives rise to the requirement for the guanine [the base paired with (-)-C5] in the sequence 5'-CAAGTG-3' and therefore is a primary determinant of the nucleotide sequence of the vnd/NK-2 homeodomain binding site in DNA. Mutation of this base pair reduces the protein-DNA affinity by a factor of 4-25. Guanine in this position is distinct from all other homeodomain-DNA complexes whose structures have been solved thus far, with thymine being most common. The hydrophobic effect provides the principal mechanism favoring the contact between Y54 and (-)-C5. Mutation of this tyrosine to methionine (a conservative substitution) results in a 10-fold reduction in the binding affinity of the homeodomain to its cognate DNA. The functional consequences of the replacement of tyrosine by methionine have been investigated in transgenic *Drosophila* experiments. All transgenic Drosophila lines with the *vnd/NK-2* gene that encodes for methionine rather than tyrosine in position 54 of the homeodomain and with the endogenous *vnd/NK-2* gene eliminated are embryonically lethal. These results demonstrate the close relationship between sequence specific DNA binding and capacity of the homeodomain containing transcription regulator to function properly.

The N-terminal arm of the homeodomain is inserted into the minor groove of the DNA and makes base specific contacts. Leucine-7 of the vnd/NK-2 homeodomain is important for sequence specific DNA recognition and is, in fact, conserved for the NK-2 class of homeodomains. Furthermore, L7 of the vnd/NK-2 homeodomain appears to be important in sequence specificity in a manner not observed in any other homeodomain-DNA complex. L7 is located above the minor groove and packs tightly against the (+)A3 ribose ring and is close to the adjacent (+)C2 (-)G2 base pair. This latter interaction with L7 gives rise to the preference of the vnd/NK-2 homeodomain for the C in position one of the 5'-CAAGTG-3' DNA consensus sequence. An L7A mutation results in a 10-fold decrease in the affinity of the vnd/NK-2

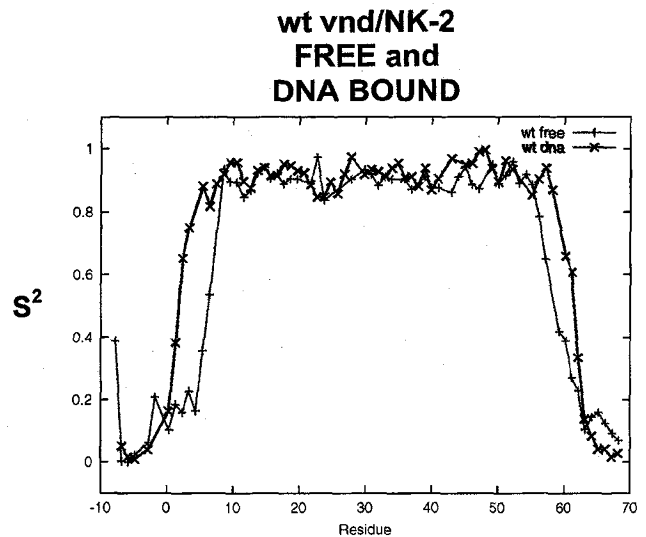

Fig. 5. Comparison of the order parameters, S^2, for the wild type vnd/NK-2 homeodomain in the free and DNA-bound states.

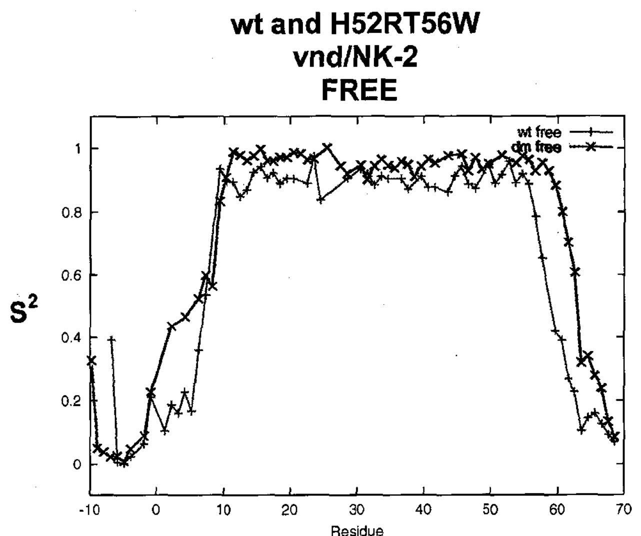

Fig. 6. Comparison of the order parameters, S^2, for the wild type and H52RT56W double mutant vnd/NK-2 homeodomain in the absence of DNA.

homeodomain for its cognate DNA. In a separate homeodomain protein, MSX2, a mutation in position 7 from proline to histidine is associated with the genetic disease craniosynostosis (Ma *et al.*, 1996). Since the structures of all of the classical homeodomains are homologous, the interaction of residue 7 with the DNA is also homologous and, thus, the molecular basis of craniosysostosis with proline-7 replaced by histidine is the change in the interaction and the alteration of the binding affinity of MSX2 to its cognate DNA.

In addition to the structural studies on the wild type vnd/NK-2 homeodomain, the ^{15}N relaxation behavior and heteronuclear Overhauser effect data were used to characterize and describe the backbone dynamics of the protein both in the free and DNA-bound states. The spin-lattice relaxation time (T_1) behavior at three distinct magnetic field strengths, in agreement with the structural studies, demonstrates that the homeodomain is structured between residues 10 and 52 in the unbound state and up to residue 60 in the DNA bound state (Fausti *et al.*, to be published). Furthermore, the relaxation data show that the N-terminal arm is significantly less mobile in the DNA-bound state, due to the interaction in the minor groove of the DNA. A comparison of the Lipari-Szabo order parameter, S^2, for the wild type vnd/NK-2 homeodomain both free and bound to its cognate DNA is given in Fig. 5. This comparison demonstrates the effect of the elongation of helix III upon binding to the DNA as well as the reduction in the motional behavior of the N-terminal arm due to the interaction with the minor groove of the DNA. In addition, the overall correlation time of the homeodomain was determined to be around 10.5 ns at 282 K for the free homeodomain and 11.5 ns at 308 K in the complex with a 16 base-pair fragment of DNA that contains the consensus sequence. One purpose of this study was to compare the dynamics of the wild type and several mutants of the homeodomain. Also, to better understand the dynamic behavior of the homeodomain and to gain insight into the role of variable residues in the C-terminal region of the DNA recognition helix, we carried out ^{15}N relaxation studies on the wild type and H52R/T56W mutant vnd/NK-2 homeodomain, both in the free and in the DNA-bound state. Although these mutations in positions 52 and 56 do not alter DNA binding affinity, arginine in position 52 forms a salt bridge with glutamate in position 17 and tryptophan in position 56 is spatially close to the hydrophobic core and to residues 19-26 in helix I and the loop region. Thus, it is of interest to examine any modifications in the motional behavior introduced by the two mutations. A comparison of S^2 for the wild type and H52RT56W double mutant homeodomains is given in Fig. 6. In addition, the relaxation behavior of both the wild type and double mutant is studied in the absence and presence of sodium phosphate, the presence of which has a significant effect on the melting temperature and the related thermodynamic parameters.

As a complement to the structural and dynamic studies on the wild type and mutants involving positions 52 and 56 of the vnd/NK-2 homeodomain, thermodynamic measurements were carried out (Gonzales *et al.*, to be published). The conformational stabilities of the homeodomains were investigated by differential scanning calorimetry and the binding to DNA was studied by isothermal titration calorimetry. The results of these studies are summarized as follows.

1. Unfolding (T_m °C)

	wt	H52R	H52RT56W
H_2O	25	29	33
50 mM PO_4	48	56	58

2. DNA Binding

$KD = 0.5 \times 10^{-9}$ mol at 298 K

$\Delta G = -11.7$ Kcal/mol at 298 K

$\Delta C_p = +42$ cal/K/mol

wt	H52R	H52RT56W
ΔH=-8.2 kcal/mol	ΔH=-10.8 Kcal/mol	ΔH=-11.8 Kcal/mol
ΔS=+13 cal/K/mol	ΔS=+3 cal/K/mol	ΔS=+8 cal/K/mol

It is interesting to note that the change in the heat capacity with temperature, ΔC_p, is positive rather than negative. Most proteins show a negative ΔC_p upon binding to DNA and this is interpreted as resulting from the hydrophobic effect or screeing of hydrophobic surface area upon binding. The origin of the positive ΔC_p of binding for vnd/NK-2 is unknown but may be related to the large number of positive charged side chains here. The other unusual feature is the decreasing values of ΔS for the mutant homeodomains relative to that for the wild type analog. Helix III, the DNA recognition helix, for the wild type analog must elongate upon binding to DNA, whereas the helix is partially or entirely elongated before binding for the mutants. This could result In a significant entropy penalty or negative contribution to DS. Thus, one would expect if the protein itself makes a significant contribution to the entropy of binding, that the entropy penalty would be smaller for these mutants so that DS would be larger (I.e., more positive). In fact, the trend is in the opposite direction. Although the reason for this behavior is unclear, it is quite likely that the contributions from ordering of solvent molecules around the free protein and the free DNA as well as around the complex make a significant contribution to the ΔS of binding.

Numerous single amino acid residue mutations in the homeodomain have been associated with altered embryonic development or genetic disease. For example, in the case of the homeodomain eve, a single R52H mutation has been shown to result in temperature dependent developmental abnormalities. It has been shown that the replacement of arginine by histidine results in a lowering of the thermal melting temperature of the homeodomain. Another mutation actually found in the vnd/NK-2 homeodomain is the replacement of alanine by threonine in position 35. This

replacement with threonine has been identified with significant transcriptional defects in *Drosophila* and early embryonic lethality. A corresponding mutation in position 35 of another homeodomain is associated with genetic disease. Alanine in position 35 is highly conserved and only alanine and serine are found in this position in all of the known homeodomains. We have shown that the mutant A35T vnd/NK-2 homeodomain is unable to adopt a folded conformation free in solution. In addition, the structure of the homeodomain in the DNA-bound state is significantly distorted over that normally found. Furthermore, the affinity of the A35T mutant homeodomain for the cognate DNA is reduced by a factor of 50. To the best of my knowledge, a substitution of the type described here that produces such a catastrophic structural result has not been reported previously. One plausible explanation for this behavior is that the wild type analog is only marginally structurally stable. If indeed this is so, then any modification that disrupts the structure would result in a protein that would be unable to fold free in solution. Thus, the structural basis of the early embryonic lethality in the mutant allele may result from three possible events. The first possible event would be the degradation of the protein by endogenous proteases. The second possibility would be related to the lower affinity for the DNA so that transcription regulation cannot occur. The third possibility is the significant structural alteration that the protein shows when bound to DNA alters protein-protein interactions in such a manner that transcriptional activiation does not occur properly. Similar mechanistic considerations would be valid for the case of an alanine to valine mutation in position 35 of a human homeodomain (de Kok *et al.*, 1995).

It is interesting to note that the four amino acid residue replacements (Fig. 3) in the NKX-2.5 homeodomain that are associated with congenital heart disease are non conservative changes. Because of the high homology between NKX-2.5 and vnd/NK-2, it is possible to identify likely structural or DNA-binding modifications that occur upon mutation. For the case of T41M, the obviation of N-capping is likely to have a significant structural effect at the beginning of helix III. Preliminary experiments strongly indicate that T41M NKX-2.5 does not adopt the appropriate three-helix structure required for proper binding to DNA. The asparagine residue in position 51 is invariant. The N51K mutation reduces the affinity of the homeodomain for its cognate DNA by 3 orders of magnitude. The R52G replacement produces a homeodomain that can no longer form a salt bridge between residues 17 and 52. This change will have a significant effect on the structural stability of the homeodomain. Tyrosine in position 54 of the NK-2 class of homeodomains is the single most important residue required for the recognition of the unusual sequence of DNA. The Y54C mutation will significantly alter the binding affinity of the homeodomain for its cognate DNA as well as favoring binding to a different sequence of DNA. In each of these four examples, the genetic disorder is strongly associated with significant alterations in the structure of the homeodomain and its ability to bind appropriately to DNA.

Conclusions

In this review, I hope to have provided an overall view of the question of studying the molecular basis of genetic disease caused by single amino acid residue modifications in proteins that are important in early embryonic development. The availability of the three-dimensional structures of the vnd/NK-2 homeodomain in the free and DNA-bound states provides for the atomic level interpretation of the effects of mutations in the homeodomain class of transcription factors. The structural information combined with dynamic measurements, thermodynamic studies provides detailed information on the solution behavior of the protein. Transgenic studies on site directed mutants helps bridge the gap between structural and functional studies that holds significant hope for a significantly improved understanding of the molecular basis of genetic disease and altered embryonic development. I believe that more detailed studies on proteins that have undergone site directed mutagenesis have the potential to provide significant insight to the design of therapies for some of the disease states mentioned here as well as other related conditions. A full understanding of the nature of genetic disease requires an understanding of the molecular level processes involved. While structural studies are not sufficient to understand genetic disease, I believe they are necessary. The hope is that such studies might, for example, provide critical information to the design of appropriate molecules to up-regulate good or wild type copies of genes and down-regulate the mutated or bad copies. Important success in this area is anticipated in the next decade.

Acknowledgments I would like to acknowledge the contribution of each and every coworker who, over the last 10 years, have turned the ideas described here into realities. The contribution of each and every one has been significantly more important than mine. These coworkers are: Dr. Marshall Nirenberg, Dr. Lan-Hsaing Wang, Dr. Mario Cinquanta, Dr. James M. Gruschus, Dr. Désirée H. H. Tsao, Dr. Solly Weiler, Dr. Bosong Xiang, Dr. Nico Tjandra, Dr. Carla Lintas, Dr. James Mack, Dr. Martin Gonzales, Dr. Ann Ginsberg, Dr. David Ornstein, Dr. W. Marston Linehan, Dr. Paul Duray, Dr. Michael Emmert-Buck, Dr. Cathy Vocke, Dr. Angelo Perico, Dr. Simone Fausti, Dr. Giovanni La Penna, Dr. Henry M. Fales, Dr. Simone Koenig, Dr. Jin-Soo Maeng, Dr. Jeung-Ho Ju, Dr. Oh Young Kwon, Dr. Hyungyung Kim, Dr. Kyoung Tai No, Mr. Kyoub Nam, and Ms. Kae-Jung Hwang.

References

Benson, D. W., Silberback, G. M., Kavanaugh-McHugh, A., Cottrill, C., Zhang, Y., Riggs, S., Smalls, O., Johnson, M. C., Watson, M. S., Seidman, J. G., Seidman, C. E., Plowdon, J.

and Kugler, J. D. (1999) Mutations in the cardiac transcription factor NKX2.5 affect diverse cardiac developmental pathways. *J. Clin. Invest.* **104**, 1567-1573.

De Kok, Y. J., van der Maarel, S. M., Bitner-Glindzicz, M., Huber, I., Monaco, A. P., Malcolm, S., Pembrey, M. E., Ropers, H. H. and Cremers, F. B. (1995) Association between X-linked deafness and mutations in the POU domain gene POU3F4. *Science* **267**, 685-688.

Gehring, W. J., Affolter, M. and Burglin, T. (1994) Homeodomain proteins. *Annu. Rev. Biochem.* **63**, 487-526.

Gruschus, J. M., Tsao, D. H. H., Wang, L.-H., Nirenberg, M. and Ferretti, J. A. (1997) *Biochemistry* **36**, 5372-5380.

Gruschus, J. M., Tsao, D. H. H., Wang, L.-H., Nirenberg, M. and Ferretti, J. A. (1999) *J. Mol. Biol.* **289**, 529-545.

Jiminez, F., Martin-Morris, L. E., Valasco, L., Chu, H., Sierra, J., Rosen, D. R. and White, K. (1995) *vnd*, a gene required for early neurogenesis of *Drosophila*, encldes a homeodomain protein. *EMBO J.* **14**, 3487-3495.

Ma, L., Golden, S., Wu, L. and Maxson, R. Hum. (1996) The molecular basis of Boston-type craniosynostosis: the Pro 148 His mutation in the N-terminal arm of the MSX2 homeodomain stabilizes DNA binding. *Hum. Mol. Genet.*, **5**, 1915-1920.

Kasahara, H., Lee, B., Schott, J-J., Benson, D. W., Seidman, J. G., Seidman, C. E. and Izumo, S. (2000) Loss of function and inhibitory effects of human CSX/NKX2.5 homeoprotein mutations associated with congenital heart disease. *J. Clin. Invest.* **106**, 299-308.

Kim, Y. and Nirenberg, M. (1989) *Drosophila* NK-homeobox genes. *Proc. Natl. Acad. Sci. USA.* **86**, 7716-7720.

McGinnis, W. and Krumlauf, R. (1992) Homeobox genes and axial patterning. *Cell* **68**, 283-302.

Scott, M. P., Tamkun, J. W. and Hartzell, G. W., III (1989) The structure and function of the homeodomain. *Biochim. Biophys. Acta* **989**, 25-48.

Tsao, D. H. H., Gruschus, J. M., Wang, L.-H., Nirenberg, M. and Ferretti, J. A. (1995) The three-dimensional solution structure of the NK-2 homeodomain from *Drosophila. J. Mol. Biol.* **251**, 297-307.

Weiler, S., Gruschus, J. M., Tsao, D. H. H., Yu, L., Wang, L.-H., Nirenberg, M. and Ferretti, J. A. (1998) Site-directed mtations in the vnd/NK-2 homeodomain. *J. Biol. Chem.* **273**, 10994-11000.

Xiang, B., Weiler, S., Nirenberg, M. and Ferretti, J. A. (1998) Structural basis of an embryonically lethal single Ala Thr mutation in the vnd/NK-2 homeodomain. *Proc. Natl. Acad. Sci. USA.* **95**, 7412-7416.