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Localized Structural Effects of Electrostatic Interactions in a Thermostable Enzyme

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The sequence and resolved structure of thermotrophic isopropylmalate dehydrogenase (IPMDH) and a related protein, mesotrophic isocitrate dehydrogenase (IDH), were compared emphasizing clusters of charged residues identified from X-ray crystallographic studies (Wallon, G., Kryger, G., Lovett, S. T., Oshima, T., Ringe, D., and Petsko, G. A. (1997) *J. Mol. Biol.* 266, 1016–1031). Mesotrophic isocitrate dehydrogenase was used for comparison because crystallographic data for a mesotrophic form of IPMDH was not available in the database. The structural features in the region of these clusters were compared and localized conformational differences were found in the thermotroph compared to the mesotroph. Because the overall topology of the two proteins is similar, it was concluded that these localized structural differences induced by electrostatic interactions between charged residues in the thermotrophic enzyme were responsible for the enhanced thermal stability of proteins from thermotroph. © 1999 Academic Press

Key Words: **enzyme structure and mechanisms; cellular regulation; phosphorylation and dephosphorylation.**

Much work has been done in the field of thermostable enzymes to identify the chemical basis of thermostability. Ionic interactions have been identified as contributing to thermostability in proteins including various forms of 3-isopropylmalate dehydrogenase (IPMDH; reference 1). This is not surprising as electrostatic effects have been suggested as contributing factors to general protein stability. For example, inductive effects have been proposed to contribute to the stability of collagen (2). The replacement of hydroxyproline with the more electronegative fluoroproline increased the thermostability of collagen. The electron-withdrawing groups induced a dipole that favored enhanced thermostability consistent with the importance of electrostatic interactions in increased thermostability. Studies of other examples including thermolysin (3) and β-glycosidase (4) have identified salt bridges as contributing factors to protein stability.

Thermus thermophilus IPMDH (T-IPMDH) possesses more salt bridges than its mesotrophic form, and the salt bridges seem to form extensive and elaborate networks (1). Residues involved in these interactions form discrete clusters, consisting of 2 to 5 residues. Molecular modeling was also used to predict a structure for a less thermostable IPMDH from a psychrophilic (cold-loving) bacterium which demonstrated the presence of fewer salt bridges (5). Comparison of thermotrophic and mesotrophic isozymes of glutamate dehydrogenase also provided results consistent with ionic interactions supporting enhanced thermostability (6). These authors found a correlation between thermostability and balanced charge density with mesotrophic isozymes having a decrease in the balance of charged residues. These data collectively supported a role for electrostatic interactions in thermostability.

IPMDH is related to IDH and both have been characterized in a variety of organisms, including *E. coli* and *T. thermophilus.* Many key residues involved in substrate interactions and other functions are conserved, with 37% sequence identity between T-IDH and E-IDH (7) and 33% sequence identity between T-IDH and T-IPMDH. IDH and IPMDH have similar structures and similar reaction mechanisms (8,9) except for a difference in substrate specificity. Magyar et al. (10) had predicted more ion pairs and increased stability of alpha helical structures in T-IPMDH from modeling studies based on a comparison of E-IDH and T-IPMDH. Comparison to the preliminary structure of the E-IPMDH provided similar conclusions, showing that comparisons of E-IDH and T-IPMDH is a valid approach for developing insight into these differences between mesotrophic and thermotrophic proteins. Because detailed X-ray crystallography structures of mesotrophic IPMDH and thermotrophic IDH (11) were not available in the databases, IPMDH (T-IPMDH)

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TABLE I

Residues in Cluster Regions

Cluster	T-IPMDH	E-IDH
1	Glu17, Lys21, Arg24	Pro43, Lys47, Asp50
2	Lys159, Glu163, Glu201	Glu203, Lys207, Gln247
3	Glu171, Arg174, Lys178, Asp208	Glu215, Ile218, Asp223, Glu274
4	Asp184, Arg196, Glu200, His213	His229, Lys242, Tyr246, Asp279
5	His179, His222, Arg229	Ser224, Glu288, Glu295
6	Glu299, His300, Arg309, Glu312	Arg365, His366, Leu375, Lys378
7	Glu321, Arg342, His343	Lys387, Glu407, Phe408

Note. Indicated residues were chosen based on the analysis of Wallon et al. (1), and comparison of sequences using the re-sequence alignment of Miyazaki et al. (7) as corrected by Kirino et al. (12).

from *T. thermophilus* and IDH (E-IDH) from *E. coli* were compared for insight into the structural effects of charged residues (12).

EXPERIMENTAL PROCEDURES

Structural information for T-IPMDH and E-IDH was downloaded from the Brookhaven Protein Database using the World Wide Web. The coordinate files had the Brookhaven designations 1HEX and 9ICD, respectively. Both enzymes included bound nucleotide, NAD^+ or $NADP^+$. A variant of E-IDH (5ICD) with bound Mg and isocitrate showed similar results. Structures were viewed and oriented using the program RasMol on a Motorola StarMax 3000/160 computer (Macintosh clone). The clusters examined included the residues shown in Table I.

RESULTS AND DISCUSSION

The sequence alignment published by Miyazaki et al. (7), as corrected according to Kirino et al. (12), was renumbered according to the sequence of E-IDH and is shown in Figure 1. Select residues, including residues SKPEV (numbers 181 to 185) of T-IPMDH, were re-aligned after initial comparison of protein structures. The cluster analysis of T-IPMDH by Wallon et al. (1) was used to choose residues involved in electrostatic interactions. Seven cluster groups containing at least three residues were identified. Comparing these data with the sequence alignment revealed residues not conserved in the mesotroph. These residues were considered for analysis because they were defined positions which were charged residues in the thermotrophic enzyme, but not in the mesotroph.

Consideration of Charge Clusters

The identified cluster groups were designated Groups 1 to 7. In Group 1, T-IPMDH has the following residues: E43, K47, and R50. T-IDH has E43, K47, and E50. The mesotroph, E-IDH, has the residues P43,

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T-IPMDH                                MKVAVLPGDGIGPEVTEAALKVLRALDEAEGGPT
T-IDH                PLITTETGKKMHVLEDGRKLITVIPGDGIGPECVEATLKVLEAA--KAP---
E-IDH          MESKVVVPAQGKKITLQAGKLNVPENPIIPYIEGDGIGVDVTPAMLKVVDAAVEKAYKGE   60

T-IPMDH        --YEVFRFGGAAIDASA-PFPEPTRKGVEEAEAVLLGSVGGPRLGRPSPQDPPGDGASFL
T-IDH          LAYEVRE-AGAS--VFRRGIASGVPQETIESIRKTRVVLKGPLETPVGYGEK--SANVTL
E-IDH          RKISWMEIYTGEKSTQVYGQDVWLPAETLDLIREYRVAIKGPLTTPVGGGIR--SLNVAL  118

T-IPMDH        KEKPDLFANLRPAKVFPGLERLSPLKEEIARGVDVLIVRELTGGIYFGEPRGM--SEAE-
T-IDH          RKLFETYANVRPVREFPN----VPTPYAG-RGIDLVVVRENVEDLYAGIEHMQTPSVAQT
E-IDH          RQELDLYICLRPVRYYQG----TPSPVKHPELTDMVIFRENSEDIYAGIEWKAD-SADAE  173

T-IPMDH        -AWNTERY-----------------------SKPEVERVARVAFEAAR-KRRKHVVSVDK
T-IDH          LKLISWKG-----------------------S----EKIVRFAFELARAEGRKKVHCATK
E-IDH          -KVI--KFLREEMGVKKIRFPEHCGIGIKPCSEEGTKRLVRAAIEYAIANDRDSVTLVHK  230

T-IPMDH        ANVLEVGE-FWRKTVEEVGR--------------------GYPDVALEHQYVDAMAMHLV
T-IDH          SNIMKLAEGP-KRAFEQVAQ--------------------EYPDIEAVHIIVDNAAHQLV
E-IDH          GAIMKFTEGAFKDWGYQLAREEFGGELIDGGPWLKVKNPNTGKEIVIKDVIADAFLQEIL  290

T-IPMDH        RSPARFDVVVTGNIFGDIG-NLRADLPGSLGLLPSASLGRGTPVFEPVHGSAPDYAGKGR
T-IDH          KRPEQFEVIVTTNMNGDILSDLTSGLIGGLGFAPSANIGNEVAIFEAVHGSAPKYAGKNV
E-IDH          LRPAEYDVIACMNLNGDYISDALAAQVGGIGIAPGANIGDECALFEATHGTAPKYAGQDK  350

T-IPMDH        -NPTAAILSAAMMLEQLRPGG-LARKVEDA-AKALLE--TPPPD-LG-GSAA----REAF
T-IDH          INPTAVLLSAVMMLRYLEEFATADLIENALLYTLEEGRVLTGDVVGYDRGAKT---TE-Y
E-IDH          VNPGSIILSAEMMLRHMGWTEAADLIVKGMEGAINAKTVTYDFERLMD-GAKLLKCSE-F  408

T-IPMDH        TATVLRHLA-----------------------------------------------------
T-IDH          TEAIIQNLGKTPRKTQVRGYKPFRLPQVDGAIAPIVPRSRRVVGVDVFVETNLLPEALGKA
E-IDH          GDAIIENM------------------------------------------------------ 416
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FIG. 1. Amino acid sequence alignment E-IDH, T-IDH, and T-IPMDH. Shown is an alignment of the corrected sequence of Kirino et al. (12) based on the initial alignment presented by Miyazaki et. al. (9). Based on the described structural comparison, some residues in T-IPMDH have been shifted and are underlined for identification. A portion of the carboxyl-terminal region of T-IDH has been omitted for clarity.

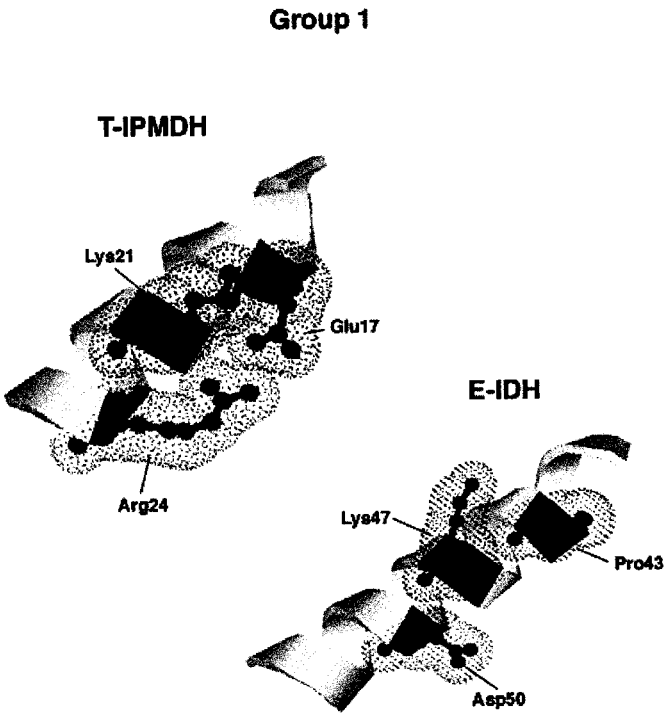

FIG. 2. Comparison of Cluster 1. Shown is a comparison of the helices containing residues in cluster 1 from E-IDH and T-IPMDH. The alpha helix in the thermotrophic enzyme, but not in the mesotroph, is slightly bent to accommodate the interaction of the three charged residues.

K47, and D50. As shown in Figure 2, these residues are all found in a single alpha helix and the presence of these charged residues has a significant effect on the structure of the helix. In E-IDH, these residues seem to be positioned in the helix with the side chains oriented away from each other. In T-IPMDH, these residues interact electrostatically and seemed to cause a distortion in the helix. The interaction involves both positively charged residues which appear to be sharing the negatively charged glutamate. The double change of residues at positions 43 and 50 in the thermotrophs seem to create a 'sandwich' effect, not possible in the mesotroph (P43 and D50).

The residues in group 2 from T-IPMDH are present in two linked helices with the residues oriented toward the interior between the helices (not shown). Lys-159 and Glu-163 on the same helix seemed to interact with Glu 201 in the second helix oriented away from the Lys-159 and Glu-163. According to the alignment of Miyazaki (7), the corresponding residues in E-IDH are Arg-180, Gly-184, and Gln-247. These residues are not in proximity. Gln-247 is quite distal from Arg-180 and Gly-184. Comparison of the structures helped us identify residues in E-IDH in the same position within a helix as Lys-159 and Glu-163. These residues, Lys-207 and Glu-203, are the same residues in reversed positions. As with the residues in T-IPMDH, Lys-207 and Glu-203 seemed to be interacting. As a consequence of identifying these residues, the alignment has been adjusted with residues SKPEV, residues 181 to 185 of T-IPMDH, being repositioned and now aligned with residues 201 to 205 of E-IDH. This makes the sequence alignment consistent with the structural comparison.

In Group 3, both T-IPMDH and T-IDH have conserved residues (Figure 3). These residues are E171, R174, K178, and D208 with several differences from the sequence of E-IDH. E171 is conserved as E215, but E-IDH has I218 in place of R174, D223 in place of K178, and E274 in place of D208 as corresponding positions. In E-IDH, these residues are arranged as two pairs: Glu215 is proximal to Ile218, both found in the same alpha helix, and Asp223 is proximal to Glu274, both located at the ends of nearby beta sheets. In the mesotroph, the two negatively charged residues (D223 and E274) are spatially proximal, but antipodal and predicted to destabilize the protein (Figure 3). With the replacement of the aspartate with lysine as found in the thermotrophs, a positively charged residue is present and will become involved in an electrostatic pair. Another difference is E274 in the mesotroph compared to D274 in the thermotrophs. The change in this site may have possible steric considerations that affect electrostatic interactions in this cluster.

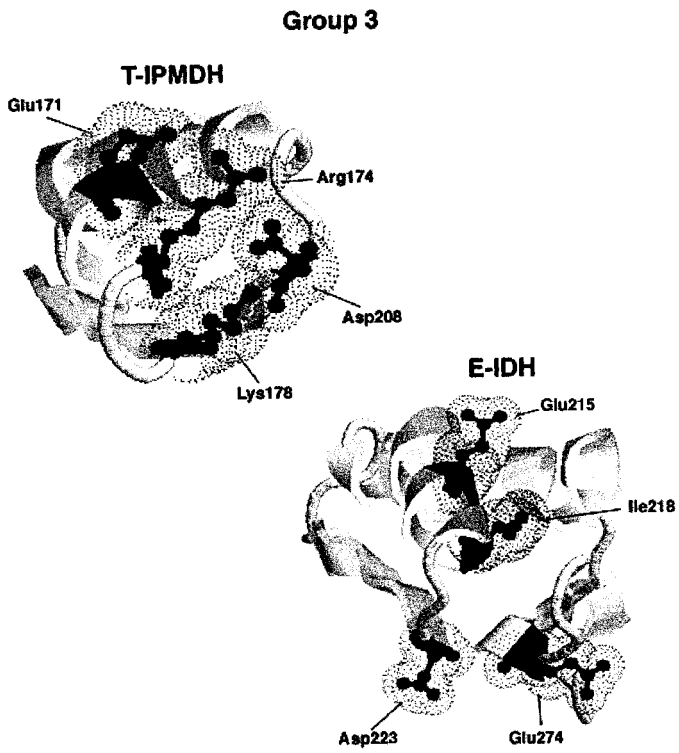

FIG. 3. Comparison of Cluster 3. Bending of the structure of the thermotrophic enzyme is evident as is the interaction between the charged residues. The loop region containing residue 178 (analogous to position 223 in E-IDH) is twisted to allow lysine-178 to participate in an ionic interaction.

Group 4 is an example of charge interactions which likely do not contribute to enhanced thermostability. Group 4 is a four-residue cluster. It is more complex than either of the other two described, because there are several differences between the proteins. The composition of the group in T-IPMDH is D229, R242, D246, and H279. In T-IDH, the residues are T-229, K242, E246, and H279. In E-IDH, the residues are H229, K242, Y246, and D279. These residues are clustered together in a diamond-like pattern (not shown) in both the mesotroph and the thermotroph. This cluster may contribute to the folding of the proteins with similar roles in all proteins in this gene family.

Group designation 5 shows greater diversity in the types of residues between T-IPMDH and E-IDH. T-IPMDH has residues His-179, His-222, and Arg-229 whereas E-IDH has residues Ser-224, Glu-288, and Glu-295 in corresponding structural positions. The difference between the arrangement of residues in T-IPMDH and E-IDH is subtle, but still detectable and relevant (not shown). The side chains of residues His-179 and Arg-229 are oriented differently, bringing them into position to interact with both His-222 and each other. In E-IDH, Glu-288 is in approximately the same orientation as His-222 in T-IPMDH. Residues Glu-295 and Ser-224, however, are not oriented to have effective interactions. The possibility of a significant charged interaction between these residues is reduced.

Cluster 6 in both proteins is composed of residues spaced the same within the primary sequence with the first 2 residues found adjacent within a helix followed by a turn and another helix having the last two residues (see Figure 4). The residues are Glu-299, His-300, Arg-309, and Glu-312 in T-IPMDH and residues Arg-365, His-366, Leu-375, and Lys-378 in E-IDH. The position of the His residue is conserved in T-IPMDH and E-IDH and found at the end of the first (upper) helix. The electrostatic interactions present only in T-IPMDH cause a change in the turn and the second (or lower) helix becomes twisted. Opposite faces of the lower helix orient toward the first (or upper) helix in the two proteins. The lower alpha helix bearing Arg-309 and Glu-312 is twisted to position the residues for interaction with Glu-299 and His-300. His-300 is also bent toward the other residues. In E-IDH, the lower alpha helix is twisted so that Leu-375 and Lys-378 face away from the residues on the upper helix. On the upper helix, His-366 and Arg-365 are oriented away from each other. There are a number of dramatic structural differences in this cluster between T-IPMDH and E-IDH.

Cluster 7 shows backbone differences related to the formation of ionic interactions (not shown). The residues are Glu-321, Arg-342, and His-343 in T-IPMDH and residues Lys-387, Glu-407, and Phe-408 in E-IDH. In both proteins, an amino terminal turn or sheet leads into a helix containing Arg-342 and His-343, or Glu-407 and Phe-408. The helix seems to be longer in T-IPMDH. Electrostatic interactions present only in T-IPMDH seem to result from a re-arrangement of the turn causing Glu-321 to be oriented toward Arg-342 and His-343. Such an interaction is not found in E-IDH, possibly because of the placement of Phe-408 in this region.

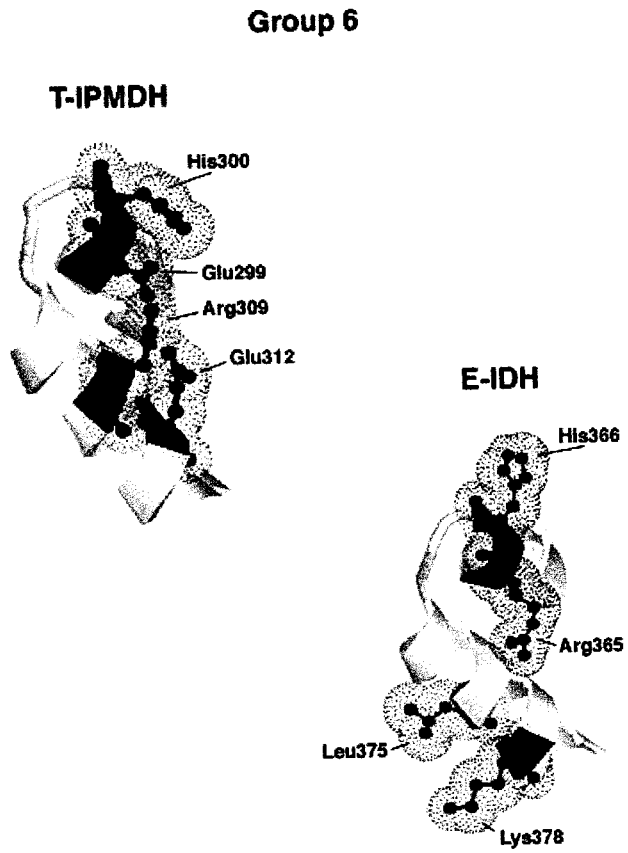

FIG. 4. Comparison of Cluster 6. The residues in this cluster are positioned on two proximal helices connected by a turn. The placement of charged residues in T-IPMDH in this cluster causes a reorientation of the turn relative to the helices. The charged residues in T-IPMDH, but not E-IDH, are able to interact between helices.

Role of Ionic Interactions

Based on comparisons of these cluster regions, ionic interactions are concluded to enhance thermostability by causing localized conformational changes in secondary structure. Many of these differences were identified in or near helical regions consistent with modeling studies (11) showing increased stability of α-helices in thermotrophic enzymes. In some cases (clusters 3 and 5, and clusters 2 and 4), adjacent residues within a helix participate in different cluster interactions providing multiple stabilizing interactions to the helix. Clusters 3 and 6 are on opposite sides of a cleft and many water molecules were bound in the cleft between

these two clusters, possibly bridging the two clusters to provide additional stabilization. We have not considered electrostatic interactions thought to be involved in subunit-subunit interactions and may have similar effects.

Summary

The structural analysis described here has identified specific regions of an enzyme family which may be critical for defining thermostability. Site directed mutagenesis at selected positions could be used to test this model. Consider cluster 1 shown in Figure 2. Both thermotrophs have glutamate at residue 43 with proline at the same position in the mesotroph. Replacing the uncharged P residue with a charged E residue would increase the likelihood of the residue being involved in an electrostatic interaction and may make the mesotroph behave more like the thermotrophs. Changing D50 to E50, as in T-IDH, changes little except the length of the residue which may be significant because the longer Glu residue may have greater flexibility and ability to interact with its proximal residues. The double mutation P43E + D50E converts this region to exactly that found in T-IDH, creating a kind of 'sandwich' effect, with Lysine 47 between the two Glutamates. The interaction involves both positively charged residues which appear to be sharing the negatively charged glutamate and could readily assist the structural differences found. The structural differences noted between the mesotrophic and thermotrophic enzyme can be tested using mutagenesis. Some work has already been accomplished using mutagenesis. For example, Akanuma et al. (13) made mutations at Ala-172 of T-IPMDH replacing this residue with Asp, Asn, Glu, and Gln. Although not affecting catalytic properties, three of the mutations did increase thermostability. Although not expected to be directly involved in any electrostatic interaction, Ala-172 is positioned in the first helix of cluster 2. Introducing polar residues at position 172 may have similar influences as the sites in the identified clusters.

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