Structural Basis of Human Triosephosphate Isomerase Deficiency

**MUTATION E104D IS RELATED TO ALTERATIONS OF A CONSERVED WATER NETWORK AT THE DIMER INTERFACE**

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Claudia Rodríguez-Almazán, Rodrigo Arreola, David Rodríguez-Larrea, Beatriz Aguirre-López, Marietta Tuena de Gómez-Puyou, Ruy Pérez-Montfort, Miguel Costas, Armando Gómez-Puyou, and Alfredo Torres-Larios

From the Departamento de Bioquímica, Instituto de Fisiología Celular, Universidad Nacional Autónoma de México, Circuito Exterior s/n, Ciudad Universitaria, Apartado Postal 70-243, Mexico City 04510, Mexico, and Departamento de Química Física, Universidad de Granada, 18071 Granada, Spain

Human triosephosphate isomerase deficiency is a rare autosomal disease that causes premature death of homozygous individuals. The most frequent mutation that leads to this illness is in position 104, which involves a conservative change of a Glu for Asp. Despite the extensive work that has been carried out on the E104D mutant enzyme in hemolysates and whole cells, the molecular basis of this disease is poorly understood. Here, we show that the purified, recombinant mutant enzyme E104D, while exhibiting normal catalytic activity, shows impairments in the formation of active dimers and low thermostability and monomerizes under conditions in which the wild type retains its dimeric form. The crystal structure of the E104D mutant at 1.85 Å resolution showed that its global structure was similar to that of the wild type; however, residue 104 is part of a conserved cluster of 10 residues, five from each subunit. An analysis of the available high resolution structures of TIM dimers revealed that this cluster forms a cavity that possesses an elaborate conserved network of buried water molecules that bridge the two subunits. In the E104D mutant, a disruption of contacts of the amino acid side chains in the conserved cluster leads to a perturbation of the water network in which the water-protein and water-water interactions that join the two monomers are significantly weakened and diminished. Thus, the disruption of this solvent system would stand as the underlying cause of the deficiency.

**Triosephosphate isomerase (TIM** or TPI, a ubiquitous, essential glycolytic enzyme, catalyzes the interconversion between glyceraldehyde 3-phosphate and dihydroxyacetone phosphate (1, 2). The functional structure of all known TIMs is assembled from monomers of about 250 residues into homodimers or tetramers. The crystal structure of the enzyme from 17 different species has been described, including the human enzyme (HsTIM) (3, 4). Despite the fact that each monomer has a full set of catalytic residues, the enzyme is only active in its oligomeric state (5, 6).

In humans, TIM deficiencies are a rare class of autosomal diseases that are clinically manifested by chronic hemolytic anemia, neuromuscular disorders, neurological alterations, and cardiomyopathy and that result in the death of affected individuals within the first 5 years of age. The deficiencies are due to mutations in the TIM gene, with the most frequent occurring in the codon for amino acid 104 that leads to the replacement of Glu by Asp (reviewed in Ref. 7). In studies of hemolysates from patients affected by TIM deficiency, the activity of the enzyme is found to be more than an order of magnitude lower than in normal individuals. Further, this reduction is accompanied by accumulation of dihydroxyacetone phosphate to levels several times higher than those of the controls (7–14). At high concentrations, dihydroxyacetone phosphate inhibits *myo*-inositol-3-phosphate synthase (15); this inhibition may be related to the clinical manifestations of TIM deficiencies. In addition, mutations in TIM may lead to the accumulation of methylglyoxal (16, 17). The binding of normal and mutant enzymes to inside-out erythrocyte membranes and microtubules has also been performed (11, 18). In these studies, it was observed that binding of the E104D and F240L mutants is higher than in the controls. The low TIM activity in TIM-deficient patients was attributed, at least in part, to this higher binding.

Studies on the characteristics of E104D HsTIM have been performed in either cell extracts from patients, in fibroblasts from patients that carry the mutation, or in cells transfected with the mutant gene (9–12, 19, 20). The data are consistent with TIM activity of affected individuals being substantially...
lower than in unaffected individuals; it was also shown that TIM activity in the E104D mutant is thermolabile (12, 20). A two-hybrid system in yeast was used to explore the interaction between the two monomers of various mutant enzymes, including E104D (21). In all cases, it was observed that there is a weakened interaction between the two monomers.

Despite extensive work that has been carried out on the E104D mutant enzyme in hemolysates and whole cells, the characteristics of the purified enzyme have not been ascertained. Since the properties of the isolated pure protein were expected to provide direct evidence as to whether intrinsic alterations of the enzyme or external factors explain the measurements made in extracts or whole cells, we determined the basic biochemical and biophysical characteristics of recombinant E104D TIM and compared them with those of the recombinant wild type HsTIM. The data indicate that the kinetic parameters of the mutant and wild type enzymes are nearly identical. Determination of the thermal denaturation profiles by circular dichroism and differential scanning calorimetry revealed that the enzyme is thermolabile. In addition, the data showed that the wild type enzyme and the mutant enzyme have different thermal denaturation routes; wild type has two transitions, whereas the mutant has only one. Measurements of the stability and reactivation of the wild type and recombinant enzymes from unfolded monomers, obtained with guanidinium chloride, show that the mutant dimers are less stable than the wild type dimers and that the formation of active dimers from unfolded monomers is lower in the mutant enzymes than in the wild type.

In regard to the atomic structure of the mutant enzyme, it is noted that the crystal structure of chicken and human TIM at 2.5 and 2.8 Å resolution, respectively, were previously used to explain the low activity that had been observed in patients affected by this mutation, which is located in the interface neighborhood of the enzyme, at ~10 Å of the active site region. It was proposed that a counterbalance of charges in the zone would lower the stability of the dimer, with an eventual perturbation of the active site (3, 20). However, we surmised that the crystal structure of the HsTIM E104D mutant would provide more accurate structural information. Indeed, the crystal structure of E104D HsTIM at 1.85 Å resolution revealed that the geometry of the catalytic residues was not modified and that the most important consequence of the E104D mutation is the disruption of a conserved water network that spans the dimer interface and appears to be essential for maintaining the stability of TIM dimers.

**Experimental Procedures**

**Cloning and Purification of Wild Type Human Enzyme and Its E104D Mutant**—The vector pARHS-HsTIM, encoding the protein sequence of wild type HsTIM, was kindly donated by Dr. Joseph Martial (Université de Liège). The HsTIM sequence was subcloned into the expression plasmid pET3b (Novagen). This vector was modified in order to introduce a His tag sequence on the amino terminus of the enzyme and, on a second round, a tobacco etch virus protease recognition sequence. In both cases, a two-stage PCR procedure was used, based on the QuikChange site-directed mutagenesis method (Novagen), as described in Ref. 22. The mutant E104D was constructed on this modified plasmid using the QuikChange protocol. The plasmids were transformed into *Escherichia coli* strain BL21(DE3) pLysS. Cells were grown at 37 °C in LB medium containing ampicillin and chloramphenicol until an A600 nm of 0.6 was reached. At that time, they were induced for 3 h with isopropyl β-D-1-thiogalactopyranoside. The same method was used to prepare the E104D HsTIM mutant.

The pellet of cells from a 2-liter culture was suspended in 20 ml of buffer A containing 50 mM sodium phosphate buffer, pH 8.0, 300 mM NaCl, and 10 mM imidazol. Cells were lysed by sonication and centrifuged at 20,000 × g for 30 min. The supernatant was loaded on a column containing 10 ml of Ni2+-nitritotriacetic acid-agarose resin (Qiagen). The protein was eluted with a linear gradient of buffer A plus 500 mM imidazole and dialyzed for 2 h against a buffer containing 50 mM Tris, pH 8.0, 0.5 mM EDTA, and 1 mM dithiothreitol. The protein was then cleaved using purified recombinant His-tagged tobacco etch virus protease expressed from the vector pRK508 (23) and purified to homogeneity. The protease was added at a ratio of 1 µg of protease per 50 µg of HsTIM and incubated at 30 °C for 18 h. The mixture was then concentrated on Amicon Ultra filters (molecular weight cut-off 10,000; Millipore) and dialyzed against buffer A. The His-tagged tobacco etch virus protease was subsequently removed by batch treatment with 4 ml of Ni2+-nitritotriacetic acid-agarose. Protein samples were >99% pure (with no His-tagged protein or other contaminants) and homogeneous, as judged by denaturing and native gel electrophoresis and mass spectrometry. Approximately 20 mg of pure protein were obtained per liter of culture. The enzymes were stored for up to 2 weeks at 4 °C in a buffer containing 100 mM triethanolamine, 10 mM EDTA (pH 7.4). Alternatively, the enzymes were precipitated with ammonium sulfate at 75% saturation and maintained at 4 °C.

**Enzyme Activity**—This was measured in the direction of glyceraldehyde 3-phosphate to dihydroxyacetone phosphate. The assay system (1 ml) contained 100 mM triethanolamine, 10 mM EDTA, 1 mM glyceraldehyde 3-phosphate, 0.2 mM NADH, and 0.9 units of α-glycerophosphate dehydrogenase (pH 7.4). Activity was calculated from the decrease of NADH absorbance at 340 nm in a Hewlett-Packard spectrophotometer with a multi-cell attachment at 25 °C. The reaction was started by the addition of enzyme, generally 2.5 or 5 ng.

**Denaturation and Reactivation of the Enzymes**—Wild type human HsTIM and its E104D mutant were denatured by incubation with 6 M guanidinium chloride for 1 h at 25 °C at a concentration of 500 µg/ml (9.2 µM dimer concentration) of 100 mM triethanolamine and 10 mM EDTA (pH 7.4). For reactivation of the enzymes, an aliquot was withdrawn and diluted at least 100-fold in 100 mM triethanolamine and 10 mM EDTA (pH 7.4). Reactivation of the enzymes was followed by the emergence of activity throughout time at 25 °C. In some cases, the reactivation reaction was determined at various protein concentrations; in all experiments, the concentration of guanidinium chloride in the reactivation mixture was 60 mM; this concentration does not affect the activity or stability of the enzymes.
Circular Dichroism—This was determined in a JASCO J-715 Spectropolarimeter (Jasco Inc.) in a 0.1-cm cell. For the spectra, 350 μg of protein/ml were placed in 20 mM MOPS, 1 mM EDTA, and 1 mM dithiothreitol. The CD signal at 222 nm was also followed as a function of temperature using a scan rate of 0.4 °C/min.

Differential Scanning Calorimetry—All experiments were done in a capillary Valery Plotnikov Differential Scanning Calorimeter microcalorimeter from MicroCal (Northampton, MA). Protein solutions were prepared by exhaustive dialysis against the buffer (100 mM triethanolamine, 10 mM EDTA, pH 7.4) that was placed in the reference cell. Protein concentrations were determined from their absorbance at 280 nm. Experiments were done at several protein concentrations (from 0.1 to 1.9 mg/ml) and scan rates (from 0.5 to 2.5 °C/min).

Size Exclusion Chromatography—Size exclusion chromatography was performed using a Superdex 200 10/300GL analytical column (GE Healthcare) on an Äkta FPLC System (GE Healthcare). 300 μl of protein at a concentration of 25 μg/ml (0.5 μM dimer) were loaded onto the column pre-equilibrated with 20 mM triethanolamine, pH 7.4, 250 μM EDTA, and 500 mM NaCl. The column was run at a flow rate of 0.5 ml/min, and absorbance was measured at 280 nm. The size exclusion column was calibrated with a gel filtration standard (Bio-Rad) containing the following globular protein markers (molecular mass and retention volumes are reported): thyroglobulin (bovine) (669 kDa, 9.8 ml), ovalbumin (chicken) (43 kDa, 15.8 ml), myoglobin (horse) (17 kDa, 17.7 ml), and vitamin B12 (1.35 kDa, 21.1 ml).

Crystallization of the E104D Mutant of HsTIM and Data Collection—The mutant E104D was crystallized via vapor diffusion by the sitting drop method. One microliter of a solution of protein at 35 mg/ml was mixed with 1 μl of reservoir solution. Initially, needle-shaped crystals were obtained in the H9 condition of the Crystal Screen HT kit (Hampton Research). Further optimization yielded better shaped crystals after 1 or 2 weeks of incubation. The best crystals were grown at 9 °C and obtained with a reservoir solution of 100 mM Tris, pH 8.5, 20% polyethylene glycol monomethyl ether 2000, 4% polypropylene glycol P400, and 10 mM NiCl2. The crystals were cryoprotected by increasing the concentration of polyethylene glycol monomethyl ether 2000 in the crystal drop to 30%; they were immediately frozen in liquid nitrogen. Diffraction data were collected at the Southeast Regional Collaborative Access Team (SER-CAT) 22-ID beamline at the Advanced Photon Source (Argonne National Laboratory), using a MarMosaic MX-300 detector. The data were processed with MOSFLM (24) and reduced with SCALA (25).

Structure Determination and Refinement—The structure was solved by the molecular replacement method with the program PHASER (26) using the coordinates of the native HsTIM at 2.2 Å resolution (4) (Protein Data Bank code 1WYI) as the search model. Refinement was made with the program CNS (27), followed by model building with COOT (28). The existence of the E104D mutation was initially confirmed by difference Fourier maps calculated using the structure of the wild type enzyme. At the start, noncrystallographic symmetry restraints were used for the refinement, using one group that included the four monomers of the asymmetric unit. The restraints were released during the last cycles of refinement, before the addition of water molecules. On all four monomers, residues 31–37 (loop 1) and 170–177 (loop 6 or flexible loop) are less clear on the electron density map. These regions are normally poorly defined in all apo-TIMs. In addition, one of the monomers (D) is somewhat more disordered than the other three (residues 130–240), probably due to lack of crystallographic contacts, but the regions that comprised the interface and active sites were well defined. Five percent of the data were used to validate the refinement. Water molecules were first located automatically in COOT and validated if a peak was observed above 3σ on a difference map and above 1.5σ on a double difference map; the molecule had to have at least one polar contact between 2.6 and 3.5 Å. σA-weighted, Fσ − Fc simulated annealing omit maps were used to further validate the quality of the model and the presence of water molecules. Data collection and refinement statistics are given in Table 1. Figures were generated with PyMOL (available on the World Wide Web).

Structure Analysis—Contacts were identified using the CCP4 programs ACT and CONTACT. The solvent-accessible surface area of water molecules was determined using AREAIMOL (25). Water molecules with an accessible surface area of less than 10 Å2 were classified as buried. Superpositions and locations of conserved water molecules were made using the program 3dss (29). Interfacial water molecules were located with the program Water Analysis Package (30). Analysis of the interfaces was made using the PISA server (31). Pocket calculation and visualization were performed with the CASTp server (32).

RESULTS

The Mutant E104D Has the Same Activity as the Wild Type Enzyme—In order to explore if the low TIM activity that is detected in hemolysates of patients that have the E104D mutation is due to an intrinsic low activity of the mutant enzyme, we determined the kinetics of wild type HsTIM and its E104D mutant. In the direction of glyceraldehyde 3-phosphate to dihydroxyacetone phosphate, the K_m values for the substrate were 0.74 mM ± 0.1 and 0.70 ± 0.15 for the wild type and the mutant enzyme, respectively, whereas the V_max values were 7080 ± 415 and 6600 ± 354 μmol/min/mg for the wild type and the E104D mutant, respectively. The two enzymes exhibited classical Michaelis-Menten behavior.

The Stability of the Dimers and the Formation of Active Dimers from Unfolded Monomers Are Lower in the Mutant E104D than in Wild Type HsTIM—Since it has been reported that in cultured fibroblasts and in erythrocytes from patients affected by the E104D mutation, TIM activity is thermolabile (12, 20), we assessed the stability of the wild type and mutant enzymes at various temperatures. The incubation of the mutant enzyme at 36 °C or above (supplemental Fig. S1A) brought about a decrease in activity that was higher than in the wild type. The loss of activity at relatively high temperatures was significantly faster in the mutant enzyme than in the wild type; for example, at 48 °C, the E104D mutant lost most of its activity.
in 4 min, whereas in the same time, the wild type retained about 40% of its activity (Fig. S1B).

The circular dichroism signals at 220 nm of the wild type and the E104D mutant were recorded as function of temperature (Fig. S2A). The E104D mutant exhibited a two-state denaturation profile with an apparent $T_m$ at 49 °C. The wild type exhibited a different denaturation pattern. In the range of 47–54 °C, a plateau that reflected a partial loss of secondary structure was observed, but as the temperature of the cell was further increased, there was a complete loss of secondary structure with an apparent $T_m$ of 60.4 °C. It is noteworthy that the CD spectra of the mutant and wild type enzymes are markedly similar (Fig. S2B).

The heat denaturation patterns of wild type HsTIM and the E104D mutant were also determined by differential scanning calorimetry. The mutant exhibited a sharp peak with a $T_m$ of 49.1 °C (Fig. 1). In contrast, the thermal denaturation profile of the wild type exhibited two transitions with $T_m$ values of 47.7 and 61.5 °C (Fig. 1). As shown in Fig. S1A, at 48 °C the wild type still retains about 40% of the initial activity, which indicates the presence of a dimeric state of the enzyme at this temperature. Thus, by differential scanning calorimetry criteria, the overall stability of the mutant enzyme is around 12 °C lower than that of wild type, which is in consonance with the CD data. No significant differences were found at other protein concentrations assayed. As the scan rate was decreased, the two transitions of the wild type and that of the mutant occurred at lower temperatures, indicating that they are all under kinetic control (data not shown). Thus, the mutation E104D causes a strong decrease in the kinetic stability. Second scans on the samples showed differential scanning calorimetry peaks that were only a minor fraction of those observed in the first scans, indicating that both the wild type and E104D mutant denature irreversibly when heated.

To examine the stability of the E104D mutant quaternary structure, we incubated the mutant and the wild type enzymes at different concentrations for 2 h at 36 °C; at that time, the specific activities of the samples was determined (Fig. 2A). Since TIM monomers are catalytically inert (5, 6, 33), it was expected that at concentrations below the association constant between monomers, there would be a drop in the specific activity of the enzyme. This phenomenon was observed in both the wild type and the mutant enzymes; however, at protein concentrations in which the activity of the mutant enzyme was hardly detectable, the wild type HsTIM still exhibited substantial activity.

### TABLE 1

| Parameters                                      | Values                                                                 |
|------------------------------------------------|------------------------------------------------------------------------|
| **Data collection statistics**                  |                                                                        |
| Space group                                     | C2                                                                    |
| Unit cell parameters (Å)                        | 320.5190 47.2871 68.9571 90.0000 97.1981 90.0000                        |
| Resolution range (Å)                            | 60.2–1.85 (1.95–1.85)                                                 |
| No. of reflections                              | 281920 (40966)                                                        |
| No. of unique reflections                      | 81960 (11447)                                                         |
| Completeness (%)                                | 93.0 (93.0)                                                           |
| $R_{	ext{int}}$ (%)                             | 10.9 (32.8)                                                           |
| I/σ                                            | 4.6 (1.9)                                                             |
| Mn(σ)/sd                                       | 9.6 (3.3)                                                             |
| No. of molecules in asymmetric unit             | 4                                                                    |
| **Refinement statistics**                       |                                                                        |
| Resolution range (Å)                            | 50–1.85 (1.92–1.85)                                                  |
| $R_{	ext{cryst}}/R_{	ext{free}}$ (%)           | 21.9 (27.5)/25.1 (30.0)                                               |
| No. of atoms, protein/solvent                   | 7372/612                                                             |
| Mean $B$ value protein/solvent (Å$^2$)          | 23.2/28.1                                                            |
| $B$ value from Wilson plot (Å$^2$)              | 18.9                                                                 |
| Root mean square deviation bond lengths (Å)     | 0.01                                                                 |
| Root mean square deviation bond angles (degrees) | 1.25                                                                |
| Cross-validated Luzzati coordinate error (5.0–1.85 Å) | 0.28                                                                |
| Cross-validated $α$ coordinate error (5.0–1.85 Å) | 0.19                                                                 |
| Residues in Ramachandran plot (%)               | 774 (92.8%)                                                           |
| Most allowed region                             | 59 (7.1%)                                                            |
| Allowed region                                  | 1 (0.1%)                                                             |
| Generously allowed region                       |                                                                        |
| Disallowed region$^a$                           |                                                                        |

$^a$ No σ cut-offs used on the refinement ($F > 0σF$).

**FIGURE 1.** Differential scanning calorimetry of the E104D mutant and wild type HsTIM. The experimental conditions are described under “Experimental Procedures.” The concentrations of the wild type and the mutant enzymes were 1.9 and 1.6 mg/ml (38 and 36 μM dimer, respectively). The dashed line depicts the thermal denaturation profile of the mutant, and the solid line depicts the thermal denaturation profile of the wild type enzymes, respectively. The scan rate was 0.5 °C/min.
The lower stability of the dimers of the E104D mutant upon dilution suggested that the mutation affects the association of monomers. Accordingly, we studied the formation of active dimers from unfolded monomers (Fig. 2B). We found that in both the mutant and the wild type enzymes, the extent of reactivation increased as the concentration of protein in the reactivation mixture was increased. However, at all of the concentrations of protein studied, the reactivation of the wild type was substantially higher than that of the E104D mutant. Because in the E104D mutant, the extent of reactivation was nearly the same in the range of 1–5 μg/ml, it is likely that in this enzyme, the association of the monomers is hindered.

The latter data suggested that in comparison with the wild type, the equilibrium between monomers and dimers in the mutant enzyme is shifted toward the former species. We explored this issue by size exclusion chromatography of the wild type and mutant enzyme after they were incubated at 48 °C. The data with the mutant showed that as the time of incubation increased, the peak that corresponded to the dimer gradually diminished; this was accompanied by the appearance of a peak with the molecular mass of the monomer (Fig. 3A). In the same conditions, the wild type only exhibited the peak that corresponded to the dimer, albeit after prolonged incubation times, a small peak that corresponded to monomeric HsTIM was detected (Fig. 3B).
The Crystal Structure of the Mutant E104D Shows no Alterations of the Active Site Region When Compared with the Wild Type Enzyme—To ascertain the molecular basis of the characteristics of the E104D mutant HsTIM, its crystal structure was solved at 1.85 Å resolution. After molecular replacement and an initial refinement, a difference map showed that the mutation was present in the four monomers of the asymmetric unit (Figs. 4A and S3A). The final maps were contoured at 1.5σ (blue) and a difference (Fo − Fe) electron density map contoured at 3σ (red). The maps were contoured at 10 Å around residue 104. The maps were obtained following the first refinement cycle made after molecular replacement on the structure of wild type HsTIM. Additional maps for the other three monomers of the asymmetric unit and omit maps around this region where the density for the solvent molecules can be seen are shown in Fig. S4. B, the conserved core of residues in the region of residue 104 mapped on the crystal structure of the mutant E104D. The two monomers are represented in cyan and green. The cluster of conserved residues is depicted in yellow sticks. C, conserved solvent molecules on the whole TIM structure. The 17 conserved water molecules detected in the mutant E104D were also observed in the presence or absence of ligands in a set of 15 different TIM crystal structures from 13 species; they are shown as red spheres.

The Crystal Structure of the Mutant E104D Shows no Alterations of the Active Site Region When Compared with the Wild Type Enzyme—To ascertain the molecular basis of the characteristics of the E104D mutant HsTIM, its crystal structure was solved at 1.85 Å resolution. After molecular replacement and an initial refinement, a difference map showed that the mutation was present in the four monomers of the asymmetric unit (Figs. 4A and S3A). The final maps were of very good quality, as indicated by inspection of simulated annealing difference omit maps (Fig. S3B). As judged by the low root mean square deviation values, there were no significant changes in the main chain of the mutant enzyme when compared with the wild type HsTIM (Fig. S4). Likewise, no differences in the general conformation of the active sites or the interface loop 3 were detected between the wild type and the E104D mutant. These observations, it should be noted, do not support the view, based on the crystal structures of chicken and human TIM, that the Glu-104 mutation could disrupt polar interactions in the region of the mutation that could ultimately lead to perturbation of the catalytic site, particularly at the level of Lys-13 and His-95 (3, 20). Here, we found no biochemical or structural evidence of such perturbations.

Residue 104 Is Central in the Formation of a Conserved Network of Buried Water Molecules—An analysis of the reported TIM structures from different species showed that residue 104 is part of a conserved cluster of residues formed by Asn-65, Asp-77, Arg-98, Glu-104, and Lys-112 between the two subunits (Figs. 4B and 5). The 10-residue cluster is present in all of the TIMs sequenced to date, except in the archeabacterial enzymes, which are tetrameric and in which none of these residues are conserved (34, 35). This conservation pattern suggests that their presence is not essential for monomer folding but may be essential for dimeric assembly and stability. In the cluster, the two subunits are oriented face to face with a distance of about 10 Å between residues 104 of the two subunits. As shown in Fig. 5, the aforementioned five residues of each of the two subunits in the wild type are linked by a set of polar interactions; however, the only direct intermolecular side chain contacts are established by Glu-77 of one subunit and Arg-98 of the other. In addition, the 10-amino acid cluster is part of the largest cavity of the enzyme, with an area of 670 Å² and a volume of 812 Å³. In each of the native TIM structures described, this cavity is filled with water molecules. A salient feature of the data in Fig. 5 is that the interactions between the amino acids forming the cluster are mediated via solvent. For example, the interactions that are made by Glu-77 with other amino acids, apart from Arg-98, are solvent-mediated (Fig. 5).

The analysis of water molecules in 15 TIMs from 13 different species at resolution of 2.4 Å or higher, including the ultrahigh 0.83 Å resolution of TIM from Leishmania mexicana (Table S1), shows a conservation pattern that matches perfectly with the cluster of conserved residues (Fig. 4, B and C). In the whole structure, 18 conserved water molecules were detected, three of which are located in the vicinity of the active site of one monomer (Table SII). The remaining 15 have an accessible solvent area of around or less than 10 Å², indicating that they are essentially buried and are all located
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**FIGURE 5.** Mutation E104D perturbs both the conserved cluster of residues and the conserved water network. Shown is a comparison between wild type HsTIM (A) and the mutant E104D HsTIM (B). The dashes represent polar interactions in the region using a cut-off of 3.5 Å. Conserved water molecules in this region are shown as yellow spheres. Values in parentheses in B indicate the number of the equivalent water molecules on the wild type enzyme (see Table SII). Nonconserved water molecules are represented as red spheres. The pairs of conserved water molecules that are related by symmetry of the dimer as numbered in the Protein Data Bank are as follows: 1wyi, 1–152, 3–222, 35–151, 40–162 and 149–163. Residues of the two different monomers are represented in cyan and green.

in the vicinity of residue 104 (Figs. 4 and 5). Except for Wat-156 (water numbering according to wild type HsTIM), the location of all of the water molecules is symmetrical, which means that there is an equivalent water molecule in each of the two monomers. It is also relevant that all of the conserved water molecules were detected in the vicinity of the interface region. Only four can be strictly classified, however, as true interfacial waters (Fig. S5 and Table SIII). None of these four water molecules are conserved in the crystal structures of monomeric TIMs, either from *Trypanosoma brucei* (Protein Data Bank codes 1MSS and 2VEI at 2.4 and 1.89 Å resolution, respectively) or from *Trypanosoma cruzi* at 2.0 Å resolution. 4 These monomeric enzymes, which are ∼1000 times less active than their dimeric counterparts, lack part of the sequence known as “loop 3,” which includes Glu-104, but they conserve the other four residues of the cluster described here. This suggests that the conserved water network is formed during the docking of the two monomers.

The E104D Mutation Weakens the Interactions of the 10-residue Cluster and Alters the Conserved Water Network—
The structures of E104D HsTIM and wild type HsTIM exhibit marked differences in the interactions between the residues and water molecules that form the highly conserved 10-residue cluster (Fig. 5B and Table 2). In contrast to Glu-104, Asp-104 in the mutant TIM does not establish contacts with Asn-65 and Arg-98. In the wild type, Glu-104 establishes two hydrogen bonds with Lys-112, whereas in the E104D mutant, there is only one, making it the main intra-

subunit polar interaction of Asp-104. As a consequence of the loss of the interaction between Asp-104 and Arg-98 and in reference to the wild type HsTIM, the latter residue was slightly displaced without manual building during the crystallographic refinement (supplemental Fig. S6). This caused an increase in the distance of Arg-98 to Glu-77 of the other subunit (Table 2), indicating that the interaction between the latter two residues is weaker than in the wild type enzyme.

A comparison of the water network in wild type HsTIM and the E104D TIM also showed important differences. In the wild type, Glu-104 and Arg-98 coordinate Wat-156; this water molecule is not present in the mutant enzyme. The contacts of Wat-162 and Wat-40 with residue 104 in the wild type are also absent in the mutant enzyme (Table 2 and Table SIV); in the wild type enzyme, the latter water molecules also contact Wat-156. The loss of the interactions correlated with a significant increase in the B-factor of both Wat-162 and Wat-40 (Table SIV). The comparison of the normalized B-factors of these conserved water molecules from all analyzed TIMs showed that none of them has values above the average of all of the detected water molecules. On the other hand, in the E104D mutant, the values are higher than the average (Table SIV). In addition, in the mutant enzyme, the contacts of the equivalent nonconserved water molecule Wat-240 with Arg-98 and Glu-77 are looser (Fig. 5 and Table 2). Finally, the equivalents of the conserved water pairs 1 with 152 and 35 with 151 on the mutant enzyme lose their contacts with Lys-112 and Arg-98, respectively (Tables 2 and SIV). In the wild type enzyme, Wat-1 and Wat-35 are at the expected distance to interact with these residues (Tables 2 and SIV).

Overall, the reduced number and weaker nature of polar contacts in the E104D mutant with the solvent molecules confer to the water network in the cluster a less dense conformation than that of the wild type (Fig. 5). Although the hydrogen bond pattern of conserved and nonconserved water molecules differs somewhat on the analyzed TIMs, the existence of a uniform compact core of interactions that link the two subunits is a constant feature in all TIMs. This core is missing in the E104D mutant. Accordingly, the structural data show that at least one of the functions of the conserved cluster of residues is to maintain a water network that is crucial in dimer stability. Apparently, the E104D mutation induces a loss of intermolecular side chain contacts that ultimately perturb the conserved network of water molecules.

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R. Arreola and A. Torres-Larios, unpublished data.
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TABLE 2
Distances between the residues that are in contact with residue 104
The contacts that are at \( \geq 3.5 \) Å are shown in boldface type, and the contacts that, in addition, differ more than 0.5 Å from the wild type enzyme are shown in underlined boldface type. WT, wild type.

| Residue and atom | Residue | Distance (WT) | Distance (E104D) |
|------------------|---------|---------------|-----------------|
| Asn-65 OD1       | Lys-112 NZ | 3.07, 2.77    | 2.94, 2.93, 3.00, 3.03 |
|                  | Cys-66 N   | 2.84, 2.98    | 2.99, 2.94, 3.12, 2.95 |
|                  | HOH 1, 152 | 2.73, 2.76    | A2074 (2.91), B2061 (2.91), C2091 (2.95), D2041 (2.97) |
|                  | HOH 216    | 2.55          | B2062 (2.82), B2072 (2.91), C2071 (3.14), C2058 (2.76) |
| Asn-65 ND2       | HOH 151, 35 | 2.89, 2.62    | A2058 (2.51), B2075 (2.58), C2098 (2.80), D2029 (2.63) |
|                  | HOH 216    | 2.55          | A2059 (2.66), B2073 (2.73), C2074 (2.71), D2030 (2.66) |
| Glu-77 OE1       | HOH 40, 162 | 2.63, 2.42    | 2.83, 2.87, 2.89, 2.8 |
|                  | HOH 149, 163 | 2.74, 2.92  | A2058 (3.50), B2075 (3.54), C2098 (3.71), D2029 (3.5) |
| Glu-77 OE2       | Arg-98 NH3 | 3.41, 3.38    | 3.89, 3.73, 3.75, 3.81 |
|                  | Arg-98 NH1 | 2.79          | 2.83, 2.87, 2.89, 2.8 |
|                  | HOH 162, 40 | 3.33, 3.66    | A2060 (3.19), A2060 (3.59), C2076 (3.25), C2076 (3.62) |
|                  | HOH 212    | 2.36          | 3.01, 3.15, 3.02, 3 |
|                  | HOH 216    | 3.31          | B2072 (3.77), B2062 (3.82), C2058 (3.53), C2071 (3.94) |
|                  | HOH 240, 240 | 3.32, 3.03   | A2060 (3.73), A2060 (3.56), C2076 (3.67), C2076 (3.51) |
| Arg-98 NE        | HOH 212    | 2.59          | 3.62, 3.79, 3.58, 4.03 |
| Arg-98 NH1       | Thr-75 O   | 2.7, 3.19     | A2074 (2.71), B2061 (2.94), C2091 (2.82), D2041 (3.03) |
| Arg-98 NH2       | HOH 35, 151 | 3.34, 3.77    | A2074 (2.71), B2061 (2.94), C2091 (2.82), D2041 (3.03) |
|                  | HOH 156    | 3.05          | 3.62, 3.79, 3.58, 4.03 |
|                  | HOH 212    | 2.66          | A2074 (2.71), B2061 (2.94), C2091 (2.82), D2041 (3.03) |
|                  | HOH 240    | 3.29, 3.18    | A2060 (3.73), A2060 (3.56), C2076 (3.67), C2076 (3.51) |
| Glu/Asp-104 OE1/OE1 | Lys-112 NZ | 2.36, 2.76    | 3.92, 3.94, 3.92, 3.94 |
|                  | HOH 1, 152 | 2.69, 2.78    | 4.45, 4.41, 4.71, 4.64 |
|                  | HOH 393    | 2.76          | 4.2, 4.0, 3.88, 4.28 |
| Glu/Asp-104 OE2/OE2 | Asn-65 ND2 | 3.46, 3.46    | 2.8, 2.94, 3.08, 2.96 |
|                  | Arg-98 NH1 | 2.81          | 3.62, 3.79, 3.58, 4.03 |
|                  | Arg-98 NE  | 3.41, 3.33    | 4.2, 4.0, 3.88, 4.28 |
|                  | Lys-112 NZ | 2.71, 3.17    | 2.8, 2.94, 3.08, 2.96 |
|                  | HOH 156    | 2.6           | 3.62, 3.79, 3.58, 4.03 |
|                  | HOH 216    | 2.6           | A2058 (4.35), B2075 (4.38), C2098 (3.9), D2029 (4.13) |
|                  | HOH 162, 40 | 3.14, 3.7    | A2074 (3.78), B2061 (3.8), C2091 (3.8), D2041 (3.90) |
|                  | HOH 201, 162 | 3.22, 3.7   | A2058 (2.92), B2075 (2.84), C2098 (2.79), D2029 (2.86) |

DISCUSSION

In this work, we characterized the recombinant E104D mutant of human TIM in order to establish how a conservative mutation that lies at the periphery of the dimer interface results in serious clinical disorders and death of affected individuals. From the known human TIM mutations, the variant E104D is the only one directly involved in intersubunit contacts. It is worth noting that either the mutant E104D or a null allele is worth noting that either the mutant E104D or a null allele is involved in all affected individuals with TIM deficiency, both homozygous and heterozygous (reviewed in Ref. 7). Several authors have reported that hemolysates of patients with the E104D mutation exhibit very low activity (9–12, 20). However, we found that the \( K_m \) and the \( V_{max} \) of the wild type and the mutant were markedly similar. Thus, it would appear that the low TIM activity in patients with the E104D mutation is not due to intrinsic alterations of its catalytic properties; instead, it seems that some other factor(s) accounts for the low activity in patients that have the mutation. In fact, one of the main characteristics of the mutant dimer is its comparatively low stability. The presented data show that at relatively high temperatures, the mutant enzyme loses activity faster than the wild type, which is in agreement with the reports in hemolysates (12) and cultured fibroblasts from patients affected by the E104D mutation (19, 20). Along this line, CD and differential scanning calorimetry data showed that heat denaturation of the wild type occurs at temperatures about 12 °C higher than in the mutant enzyme.

In this regard, it is relevant that our studies on the stability of the dimer at various protein concentrations showed that the dimers that had the E104D mutation were less stable than the wild type dimers. This is strongly suggestive that the association of the mutant monomers is lower than that of the wild type, in agreement with the double hybrid experiments of Ralser et al. (21) that show that the association of the mutant monomers is lower than in the wild type. Thus, the overall data suggested that, relative to the wild type, in the E104D mutant, the equilibrium between monomers and dimers is shifted toward the monomeric species. Indeed, it was found by size exclusion chromatography that when the mutant enzyme was incubated under conditions that bring about inhibition of activity, there was an increase in the ratio of monomers to dimers in the overall enzyme population. This phenomenon did not occur in the wild type. In vivo, this could result in the accumulation of inactive monomers. In addition, it is possible that the low activity detected in TIM deficiency patients is linked to a faster proteolytic degradation of both monomeric and dimeric mutant species due to their lower stability.

In order to gain a more detailed insight at the molecular level into the impairments of the E104D mutant dimer, we solved the x-ray crystal structure of the enzyme. The crystal structures of all of the TIMs previously described show that the residues of loop 3 (residues 65–79), which interdigitates into the opposite subunit, account for the majority of the contacts between the two subunits. However, it is observed that of the 14 residues that form loop 3, only three (Asn-65, Thr-75, and Glu-77) are strictly conserved in all of the available TIM sequences, indicating that there are no strong sequence demands in the architecture of loop 3. In this context, we want to call attention to the interfacial polar cluster formed by five residues of each of the two subunits (Fig. 5). A remarkable feature of this cluster is that,
with the exception of tetrameric TIMs, the residues that constitute this region are fully conserved in all of the 1043 sequences that were examined (Pfam entry PF00121). It is also noteworthy that of the 42 residues from each monomer that form part of the interface (calculated with the PISA server (31)), this region is the only one with such stringent sequence and structural requirements. The cluster forms a cavity that is filled with water molecules, 11 of which are conserved in all of the reported crystal structures of TIM dimers. All of the latter waters are buried (accessible surface area <10 Å²); they have low B-factors when compared with the rest of the solvent molecules that are present on all the TIM proteins, and, on average, each one of them makes more than four polar contacts with either an amino acid residue or other water molecules. The high level of conservation of amino acid residues and water molecules in the cluster in all studied dimeric TIMs, together with the data on the low stability of E104D mutant dimer, indicate that the integrity of this portion of the interface is central to the stability and overall function of the enzyme.

In the cluster, the only protein-protein interaction between the two subunits is a salt bridge linking Arg-98 and Glu-77. The importance of this electrostatic bond is illustrated by the data of Mainfroid et al. (36), which show that the R98Q mutation in HsTIM yields an inactive, presumably monomeric protein. Remarkably, the rest of the contacts that Glu-77 makes with other residues are made through water interactions. In the E104D mutant enzyme, the intersubunit Arg-98-Glu-77 salt bridge is still present, albeit the mutation brings about an increase in the distance between Arg-98 and Glu-77 that results in a weaker interaction. However, from the crystal structures of the wild type and mutant enzymes, it would seem that the principal consequence of the mutations in this region would be to alter the conserved network of water molecules that spans the two subunits of the enzyme. Specifically, in the mutant E104D, we observed the disappearance of a conserved water molecule bound to residue 104 on the wild type, an increase of the B-factor of two conserved water molecules that are linked to residue 104 on HsTIM, and the absence of several protein-solvent and solvent-solvent hydrogen bonds that are present in the wild type.

In sum, we show how a conservative change on the periphery of an intermolecular interface leads to profound changes in the formation and stability of a macromolecular complex. Specifically, by using the crystal structure of the E104D mutant and by performing a structural analysis of the 15 TIM structures from 13 different species, it was found that solvent molecules play a previously unrecognized central role in the stabilization of TIM dimers. In all likelihood, the mutation-triggered disruption of the water network is the underlying cause of the biochemical and clinical manifestations of the E104D TIM deficiency. Recently, it has been reported that mutations that are close to or form part of the dimer interface of other proteins could be associated with various diseases (37–40). Although it is known that the presence of an extensive and buried solvent interface is a common feature of protein quaternary structure (reviewed in Refs. 41–43), to our knowledge, this is the first time that a correlation between a perturbation of interfacial waters and a fatal disease has been established.

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Note Added in Proof—We have recently solved the crystal structure of the HsTIM wild-type enzyme at 1.70 Å resolution (Protein Data Bank code 2jk2). The enzyme was purified and crystallized under conditions similar to those reported here for the mutant E104D. The analysis of this structure fully supports the conclusions drawn in the present study.

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