Isopentenyl-diphosphate (IPP):dimethylallyl diphosphate isomerase is a key enzyme in the biosynthesis of isoprenoids. The mechanism of the isomerization reaction involves protonation of the unactivated carbon-carbon double bond in the substrate, but identity of the acidic moiety providing the proton is still not clear. Multiple sequence alignments and geometrical features observed in crystal structures of complexes with IPP isomerase suggest that Tyr-104 could play an important role during catalysis. A series of mutants was constructed by directed mutagenesis and characterized by enzymology. Crystallographic and thermal denaturation data for Y104A and Y104F mutants were obtained. Those data demonstrated the importance of residue Tyr-104 for proper folding of Escherichia coli type I IPP isomerase.

Isoprenoids play important roles in all living organisms; they function as steroid hormones in mammals, carotenoids in plants, and ubiquinone or menaquinone in bacteria (1). All isoprenoid compounds are characterized by the basic isoprene unit. Nature uses two activated isoprene units to build these prenoid compounds as follows: isopentenyl diphosphate (IPP) and its electrophilic allylic isomer dimethylallyl diphosphate (DMAPP) (Scheme 1). Isopentenyl diphosphate:dimethylallyl diphosphate (IPP:DMAPP) isomerase (IDI; EC 5.3.3.2) is a key enzyme involved in the biosynthesis of isoprenoids and catalyzes the isomerization of IPP into DMAPP (2). DMAPP then condenses with additional molecules of IPP to form farnesyl diphosphate, which is required for protein prenylation, cholesterol biosynthesis, and synthesis of a variety of higher molecular weight isoprenoids. Although the synthesis of IPP can occur through the mevalonate pathway in which acetyl-CoA and mevalonate are metabolites, formed upon the protonation of IPP (supplemental Scheme S1) (7). However, these studies have not yet conclusively established the origin of the proton activating the double bond in IPP. Glu-116 is likely the critical acidic residue that drives protonation by lowering the energy of the carbocation as demonstrated by the crystallographic structure of the transition-state analogue N,N-dimethyl-2-amino-1-ethyl diphosphate in complex with the enzyme (8). However, Glu-116 is probably not protonated because it is directly coordinated to the catalytic metal cation. Therefore, it could not be the proton donor. Tyr-104 is a possible candidate for proton donation in the reaction catalyzed by IDI-1. Indeed, in the crystal structures of IDI-1, Tyr-104 is located in the active site of the enzyme and is directly hydrogen-bonded to Glu-116. The precise role of Tyr-104 during catalysis is not clear and deserves special attention. The present work was designed in an effort to clarify that question.
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**Site-directed Mutagenesis**—Plasmid pYL20, which contains the idi gene from *E. coli* with an engineered C-terminal His tag (LEHHHHHHH), was available from previous studies (11). Mutations Y104A and Y104F were introduced using the QuickChange site-directed mutagenesis kit (Stratagene). The pYL20 plasmid was used as a template in all experiments.

**Purification**—*E. coli* BL21(DE3)pLysS cells (Novagen) transformed by the mutated pYL20 plasmid were grown to an OD660 of 0.4–0.5 (from OD600 of 0.2) at 37 °C. Expression of the recombinant enzymes by the mutated pYL20 plasmid were grown to an OD660 of 0.4–0.5

**Enzymatic Activity**—The principle of the IPP isomerase assay is based on the lability of DMAPP, which is transformed into the corresponding alcohol under acidic conditions, although IPP remains stable under the conditions of the assay (12). The reaction was initiated by adding the enzyme to a mixture in 50 mM Tris-HCl buffer, pH 7.4, containing the divalent metal cofactors (1.5 mM Mn2+ and Mg2+) and [1-14C]IPP 18 μM in a final volume of 50 μl. After incubation at 37 °C, the reaction was stopped by addition of 400 μl of methanol/1% concentrated HCl (4:1, v/v). The mixture was subsequently incubated for 10 min at 37 °C, and the radioactive products were extracted with chloroform. The extraction was performed by vigorous vortexing followed by centrifugation at 10,000 × g for 2 min. A 200-μl portion of the organic layer was mixed with 5 ml of a scintillation mixture (ICN Biomedicals). The radioactivity was counted as a measure for the conversion of IPP into DMAPP.

**Crystallization Experiments**—Crystallization trials were achieved by the hanging drop vapor-diffusion method at 20 °C. Each drop suspended on the top of a reservoir solution (0.6 ml) was a mixture of 4 μl of protein solution (2.0 and 3.1 mg/ml for Y104A and Y104F, respectively) with the same volume of the reservoir solution. *E. coli* idi-1 mutants precipitated when the same crystallization conditions for WT IPP isomerase were used. Crystallization parameters were therefore adapted by screening precipitant concentration (10–16% (w/v) PEG 2000 monomethyl ether (mme)), pH (4.5–6.5), and additives (ammonium sulfate, MnCl2, and MgCl2).

Small hexagonal crystals (maximum size 0.15 mm) were obtained for the Y104A mutant in 14% (w/v) PEG 2000 mme, 100 mM Tris maleate buffer, pH 4.5, in the presence of 100 mM ammonium sulfate, 20 mM MnCl2, and MgCl2. Larger crystals were obtained for the Y104F mutant under similar conditions (14% (w/v) PEG 2000 mme, 100 mM Tris maleate buffer, pH 5.0, in the presence of 100 mM ammonium sulfate and 20 mM MnCl2 and MgCl2).

Complex was obtained by soaking crystals of the Y104F mutant with 3,4-epoxy-3-methyl-1-butyl diphosphate (EIPP), a mechanism-based irreversible inhibitor (8). Solution of the inhibitor (25 mM) in Tris maleate buffer, pH 4.5, PEG 2000 mme (14%), ammonium sulfate (100 mM), MnCl2, and MgCl2. Larger crystals were obtained for the Y104F mutant under similar conditions (14% (w/v) PEG 2000 mme, 100 mM Tris maleate buffer, pH 5.0, in the presence of 100 mM ammonium sulfate and 20 mM MnCl2 and MgCl2).

**Data Collection and Structure Determination**—For the small Y104A mutant crystals (approximate size of about 0.15 mm) a complete data set to 1.76 Å was collected, after flash-freezing, at beam line BM30A (European Synchrotron Radiation Facility, Grenoble, France) on a MarResearch CCD. Data were processed with the HKL suite (13). The Y104A mutant crystallizes in space group P321 with cell parameters a = b = 71.4 and c = 61.8 Å. There is one molecule in the asymmetric unit.

For the Y104F mutant, a complete data set to 1.96 Å was collected with a Mar345 imaging plate system from MarResearch equipped with Osmic optics and running on an FR591 rotating anode generator on a single crystal. Data were processed with the MarFLM suite. The Y104F mutant crystallizes in space group P212121 with cell parameters a = b = 71.4 and c = 61.8 Å. There is one molecule in the asymmetric unit, related by 2-fold noncrystallographic symmetry.

**Diffraction data of the complex Y104F–EIPP were collected to 1.97 Å.**

**The three mutant protein solutions were concentrated by ultrafiltration (YM10, Amicon) prior to crystallization trials. In contrast to WT IPP isomerase, for which an ideal concentration to obtain crystals was adapted by screening precipitant concentration (10–16% (w/v) PEG 2000 monomethyl ether (mme)), pH (4.5–6.5), and additives (ammonium sulfate, MnCl2, and MgCl2).**

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**Diffraction data of the complex Y104F–EIPP were collected to 1.97 Å.**

**The three mutant protein solutions were concentrated by ultrafiltration (YM10, Amicon) prior to crystallization trials. In contrast to WT IPP isomerase, for which an ideal concentration to obtain crystals was about 5 mg/ml, protein solutions of the mutants could not be concentrated higher than 2.0 and 3.1 mg/ml for Y104A and Y104F, respectively, based on protein concentration estimated by UV absorption.**

**Enzymatic Activity**—The principle of the IPP isomerase assay is based on the lability of DMAPP, which is transformed into the corresponding alcohol under acidic conditions, although IPP remains stable under the conditions of the assay (12). The reaction was initiated by adding the enzyme to a mixture in 50 mM Tris-HCl buffer, pH 7.4, containing the divalent metal cofactors (1.5 mM Mn2+ and Mg2+) and [1-14C]IPP 18 μM in a final volume of 50 μl. After incubation at 37 °C, the reaction was stopped by addition of 400 μl of methanol/1% concentrated HCl (4:1, v/v). The mixture was subsequently incubated for 10 min at 37 °C, and the radioactive products were extracted with chloroform. The extraction was performed by vigorous vortexing followed by centrifugation at 10,000 × g for 2 min. A 200-μl portion of the organic layer was mixed with 5 ml of a scintillation mixture (ICN Biomedicals). The radioactivity was counted as a measure for the conversion of IPP into DMAPP.

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Data collection and refinement statistics are given in Table 1. Coordinates have been deposited at the Protein Data Bank (codes 2G73 for Y104F-EIPP complex, 2G74 for Y104F, and 1R67 for Y104A mutants).

**Table 1**

| Data collection and refinement statistics of the *E. coli* IDI-1 mutants and the complex between Y104F mutant and EIPP inhibitor |
|-------------------------------------------------------|
|                       | Y104A mutant | Y104F mutant | Y104F-EIPP complex |
| Crystal data          |              |              |                   |
| Space group           | P3,21        | P2,2,2,1     | P2,2,2,1          |
| Cell dimensions (Å)   | 71.648       | 69.264       | 68.935            |
|                       | 71.648       | 71.805       | 71.477            |
|                       | 61.327       | 91.905       | 91.955            |
| Subunits per asymmetric unit | 1 | 2 | 2 |
| Data set             |              |              |                   |
| Wavelength (Å)        | 0.9797       | 1.54179      | 1.54179           |
| Highest resolution (Å)| 1.76 (1.80-1.76) | 1.96 (2.03-1.96) | 1.97 (2.03-1.97) |
| Total reflections     | 31,357       | 33,399       | 33,019            |
| Unique reflections    | 16,594       | 33,110       | 32,213            |
| Observed reflections  | 14,173       | 30,195       | 28,124            |
| Complementarity (%)   | 89.9 (93.5)  | 99.3 (93.8)  | 99.1 (92.4)       |
| Rmerge (%)            | 4.0 (16.9)   | 5.4 (22.6)   | 5.5 (25.0)        |
| I/σ(I)                | 8.2          | 5.1          | 6.2               |
| Refinement            |              |              |                   |
| Resolution range (Å)  | 10-1.76      | 10-1.96      | 10-1.97           |
| No. of protein atoms  | 1208         | 2828         | 2820              |
| No. of ligand atoms   | 1            | 4            | 64                |
| No. of water molecules| 132          | 166          | 115               |
| Rmerge (observed/all data) (%) | 19.0 / 20.7 | 20.1 / 21.2 | 19.7 / 21.3       |
| Rfree (%)             | 23.56        | 25.32        | 25.43             |
| Root mean square deviations | Bond lengths (Å) | 0.006 | 0.021 | 0.030 |
|                        | Bond angles (Å) | 0.023 | 0.022 | 0.023 |
| Average R-factor      | 26.40        | 26.87        | 29.57             |
| All protein atoms (Å²)| 24.55        | 24.65        | 28.25             |
| All water molecules (Å²)| 36.28 | 28.80 | 29.71 |
| Ramachandran plot (%) | 99.3         | 97.6         | 98.5              |
| Core region (%)       | 83.8         | 77.5         | 77.5              |

* Statistics for the highest resolution shell are given in parentheses.
* Free test subset represents 10% of total unique reflections.
* As defined by SHELXPRO, Gly and Pro residues excluded.

**Table 2**

| Kinetic parameters of wild-type and mutant *E. coli* IDI-1 |
|----------------------------------------------------------|
| WT enzyme | Y104A mutant | Y104F mutant |
|------------|--------------|--------------|
| Relative activity (%) | 100 ± 6.6 | 0.1 ± 0.1 | 0.8 ± 0.3 |
| Kₘ (μM) | 9.5 ± 4.3 | 22.5 ± 9.9 | 14.2 ± 2.1 |
| Vₙₘₐₓ (mM·min⁻¹·h⁻¹) | 260.7 ± 43.5 | 0.1 ± 0.04 | 2.9 ± 0.02 |

Data collection and refinement statistics are given in Table 1. Coordinates have been deposited at the Protein Data Bank (codes 2G73 for Y104F-EIPP complex, 2G74 for Y104F, and 1R67 for Y104A mutants).

**Thermal Stability Evaluation**—Stability of the studied proteins versus temperature (dV/dT) was evaluated according to the solvent accessibility to aromatic residues (tyrosine and tryptophan) method (17, 18). The absorption spectra at 280 nm were obtained using an UVikonXS spectrophotometer from Bio-Tek Instruments. The denaturation studies were carried out in the temperature range 25–80 °C using a scan rate of 1 °C/min. Samples were prepared by mixing protein solutions with 50 mM Tris-HCl buffer, pH 7.4, containing the divalent metal cofactors (1.5 mM Mn²⁺ and Mg²⁺) in order to obtain a final protein concentration of 0.3 mg/ml. The solutions were then equilibrated for 10 min prior to carrying out the thermal denaturation study. A spectrum of the buffer only was recorded as a blank. Finally, the first derivative of absorbance with respect to temperature (dA/dT) was calculated in order to determine the Tₕ (temperature of protein denaturation). This Tₕ is related to the thermal stability of the protein (19).

**RESULTS**

Construction and Characterization of Tyr-104 Mutants—In order to test the importance of residue Tyr-104 in the mechanism of IDI-1, two mutants were constructed by site-directed mutagenesis and sequenced, Y104A and Y104F. In the first mutant, the phenol lateral chain of Tyr-104 is replaced by a nonaromatic side chain. In the second mutant (Y104F), the aromatic ring is conserved, but the hydroxyl group is removed.

The corresponding C-terminal His-tagged proteins were overexpressed in *E. coli* and purified by affinity chromatography. This protocol yielded mutants in a pure form suitable for enzymatic activity tests and protein crystalization. The affinity purification step (nickel-Sepharose) was introduced in order to separate the recombinant (mutated) enzymes from the constitutive chromosomic *E. coli* IDI-1 and therefore to avoid interference in the enzymatic assays.

Enzymatic activity of the two Tyr-104 mutants is greatly affected when compared with the wild-type enzyme as illustrated in Table 2. Replacement of the lateral chain of Tyr-104 by an alanine (Y104A) or a phenylalanine (Y104F) resulted in a dramatic decrease of enzymatic activity, with the mutants presenting less than 5% residual specific activity. Efficacy of the mutants is significantly reduced by a factor of 100 (Y104F, Vₙₘₐₓ = 2.9 versus 260.7 mM·min⁻¹·h⁻¹ for the WT enzyme) to 1000 (Y104A, Vₙₘₐₓ = 0.14 versus 260.7 mM·min⁻¹·h⁻¹ for the WT enzyme). This lowered activity clearly indicates that Tyr-104 is a key amino acid for the isomerization of IPP into DMAPP. In addition, determination of Kₘ values shows that affinity of both Tyr-104 mutants to the substrate is affected by the deletion of the hydroxyl moiety (Y104A and Y104F, Kₘ = 22.5 and 14.2 versus 9.5 μM for the WT enzyme). It is therefore assumed that Tyr-104 plays a role in the substrate stabilization and the proper folding of the binding site.

Crystallographic Structure of Y104A and Y104F Mutants of IDI-1—Crystals from the two mutants were grown using the hanging drop method. Crystals of the Y104F mutant were obtained in the presence of divalent cations. Their shape is similar to the one obtained with the WT enzyme (Fig. 1A), which belongs to the orthorhombic P2₁2₁2₁ space group.
group \((a = 69.1, b = 72.2, \text{ and } c = 91.6 \text{ Å})\). Crystals of the Y104A mutant appeared in similar crystallization conditions. In contrast to the Y104F protein, they exhibit a prismatic shape and belong to trigonal \(P_3_2_1\) space group \((a = b = 71.4, c = 61.8 \text{ Å})\) as the crystallographic structure of WT enzyme without any cation (Fig. 1, D and C, respectively).

First, we refined the crystallographic structure of Y104F protein to 1.96 Å. The N-terminal residues fold into a small \(\beta\)-sheet of two anti-parallel strands as shown in Fig. 1B. This folding leads to the formation of the first metal (\(M^{2+}\))-binding site that coordinates His-25 and His-32 and brings them close to His-69, Glu-114, and Glu-116. A second metal site coordinates the carbonyl group of the highly conserved Cys-67 residue, the side chain oxygen (O-\(\epsilon_2\)) of Glu-87, and four water molecules to form an MO\(_6\) octahedral coordination sphere.

Second, the structure of Y104A was refined to 1.76 Å (PDB code 1r67). No electron density is associated with the lateral chain of residue 104, consistent with replacement of the tyrosine by an alanine introduced by site-directed mutagenesis. The \(M^{2+}\) metal-binding site is absent in this structure, but \(Mg^{2+}\) is still present and is bound to Cys-67, Glu-87, and four water molecules (Fig. 1D). A comparison of the two mutants against the WT enzyme is presented in Table 3.

**TABLE 3**

Comparison of the general fold of wild-type (metal-bound and metal-free) \(E. coli\) IDI-1 against the mutated proteins (Y104A and Y104F)

|                | WT metal-bound | WT metal-free | Y104F mutant | Y104A mutant |
|----------------|----------------|---------------|--------------|--------------|
| PDB code      | 1hx3           | 1hzt          | 2g74         | 1r67         |
| General fold   | Completely folded, N-terminal region folded in \(\beta\)-sheet | N-terminal region unfolded | Absent | N-terminal region unfolded |
| \(M^{2+}\)-binding site | MO\(_6\) coordination, His-25, His-32, His-69, Glu-114, and Glu-116 | Absent | MO\(_6\) coordination, His-25, His-32, His-69, Glu-114, and Glu-116 | Absent |
| \(Mg^{2+}\)-binding site | MO\(_6\) coordination, Cys-67, Glu-87, and 4 water molecules | MO\(_6\) coordination, Cys-67, Glu-87, and 4 water molecules | MO\(_6\) coordination, Cys-67, Glu-87, and 4 water molecules | MO\(_6\) coordination: Cys-67, Glu-87, and 4 water molecules |

FIGURE 1. Comparison of crystallographic data of WT and mutants of \(E. coli\) IPP isomerase. A, metal-bound wild-type enzyme. B, Y104F mutant; Glu-114 is not represented for the clarity of the picture. C, metal-free wild-type enzyme. D, Y104A mutant. Red spheres are water molecules. These figures were produced using PyMOL (20).
Finally, a crystal of Y104F was soaked in order to obtain a complex between the mutated protein and EIPP, a mechanism-based irreversible inhibitor. Structure was refined to 1.97 Å. The general fold of the enzyme is similar to the one observed for the WT enzyme. Two non-bridging oxygens in the diphosphate moiety, the carbonyl group of the highly conserved Cys-67 residue, the side chain oxygen (O-e2) of Glu-87, and two water molecules coordinate the second metal, involved in the stabilization of IPP, to form the same MO6 octahedral coordination sphere.

Thermal Stability of the Mutated Proteins—Thermal denaturation of the enzymes was studied in order to compare the stability of the WT enzyme versus the mutated proteins. The thermal denaturations of the WT enzyme and the studied mutants were irreversible, under the experimental conditions used.

Temperature of denaturation of the enzyme decreased by 20 °C when Tyr-104 is mutated into alanine (Td, 69 and 48 °C for WT and Y104A enzymes, respectively). Stability of the enzyme is less affected when the aromatic moiety is conserved (Td, 69 and 55 °C for WT and Y104F enzymes, respectively) clearly showing the importance of the lateral OH group of this residue (Fig. 2).

DISCUSSION

Multiple sequence alignments obtained by ClustalW (version 1.74) (21) show that a series of amino acids is strictly conserved among type 1 IDIs. Beside the conserved catalytic cysteine (Cys-67 in E. coli) and glutamic acid (Glu-116 in E. coli) residues, a few other amino acids are conserved in all aligned sequences (supplemental Fig. S1). Among those residues, His-25, His-32, His-69, Glu-114, and Glu-116 are part of the first (catalytic) metal cation-binding site (6, 7). Residues Lys-21, Arg-51, Lys-55, and Arg-83 are involved in the stabilization of the diphosphate moiety of the ligand. Interestingly, Tyr-104 is another highly conserved residue (8–10). In the crystal structures reported so far for E. coli IPP isomerase, the side chain of Tyr-104 is hydrogen-bonded to the catalytic glutamate Glu-116 (6–10).

After mutation of Tyr-104 into alanine (Y104A) and phenylalanine (Y104F), we observed a reduced enzymatic activity (decreased V_m) and ligand affinity (increased K_m). These results clearly indicate that Tyr-104 is a key amino acid for the isomerization of IPP into DMAPP and could play a role in the stabilization of the substrate into the binding site.

For a second time we tried to validate this assumption by studying the crystallographic structure of the mutated proteins. The Y104F mutant presents a fold similar to the wild-type enzyme in the presence of metal cations. Therefore, mutation does not seem to affect the global conformation of the protein. Interestingly, a crystal form of the Y104A mutant, although obtained in the presence of divalent cations, is similar to the one obtained for the metal-free wild-type E. coli IDI-1 (PDB code 1hzt) (7). In this structure, the first 32 amino acids are not visible in the electron density maps revealing flexibility of this domain when the catalytic metal-binding site is not properly folded (Fig. 1D). A similar disorder of the N-terminal domain appears in the structure of the Y104A mutant. Therefore, mutation of Tyr-104 into an alanine seems to decrease the stability of the catalytic metal-binding site, even in the presence of metal cations in the solution (supplemental Fig. S2).

To measure the stability of the mutants in comparison to the WT enzyme, thermal denaturation studies were carried out by UV spectroscopy (Fig. 2). The temperature of denaturation for Y104A and Y104F is significantly lowered compared with the WT value (Td, 69, 55, and 48 °C for WT, Y104F, and Y104A enzymes, respectively), suggesting that Tyr-104 is implied in the overall stability of IDI-1 folding as proposed by the crystallographic structure of Y104A.

Based on this work, the role of Tyr-104 seems to be mainly structural. In order to check the conservation of the catalytic process, diffraction data were collected on a complex between Y104F mutant and a mechanism-based irreversible inhibitor, EIPP. The experimental electron density shows a covalent bond between one oxygen atom O1 of Glu-116 in the active site and carbon atom C-3 of the inhibitor (Fig. 3). Thus, the epoxide in EIPP reacted with a proton in order to be activated. This intermediate was then opened by Glu-116 (Scheme 2). Moreover, the
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CONCLUSIONS

The acidic moiety providing the proton during isomerization of IPP into DMAPP catalyzed by IDI-1 is not yet identified. Multiple sequence alignments and geometrical features observed in crystal structures of complexes with E. coli IDI-1 suggest that Tyr-104 could play this important role during catalysis. Therefore, two mutants (Y104A and Y104F) were constructed by site-directed mutagenesis and purified. Enzymatic characterization of those mutants shows that over 95% of activity is lost when compared with the wild-type enzyme. Moreover, affinity (Km) for the substrate is significantly affected for Y104A, and efficacy (Vmax) of the mutants is reduced by a factor of 100 (Y104F) to 1000 (Y104A).

On the other hand, crystallographic data collected on the Y104A mutant show that the first 30 N-terminal amino acid domain is flexible and not properly folded. Replacement of the lateral chain of Tyr-104 by a methyl group (Y104A) prevents formation of the hydrogen bond with the side chain of Glu-116, and as a result stability of the active site is decreased. Indeed, thermal denaturation studies show a decrease of denaturation temperature (Tdn 48 versus 69 °C for WT). A reasonable explanation for the dramatic decrease of activity of the IDI-1 Y104A mutant would be that Tyr-104 plays a structural role and maintains the active conformation of the protein by allowing proper folding of the catalytic metal-binding site.

Finally, in the crystal structure of the Y104F mutant in complex with EIPP, the enzyme is fully folded. This leads to a catalytic metal-binding site properly structured. In addition, the epoxide of EIPP was activated by a proton and then opened by Glu-116, showing that the catalytic machinery is still intact when the protein is properly folded. Although this protein is correctly folded, the Tdn value was decreased by 14 °C compared with the WT enzyme (55 versus 69 °C). This assumed that H-bonding between Tyr-104 and Glu-116 is important for the general stability of the protein.

Taken together, these structural features suggest a structural role of Tyr-104. Tyr-104 is therefore not the proton donor in the mechanism of isomerization of IDI-1. This role has to be assigned to another residue or a water molecule. Other residues possibly involved in the protonation step of isomerization of IPP by IDI-1 are now under investigation.

REFERENCES

1. Sacchettini, J. C., and Poulter, C. D. (1997) Science 277, 1778–1789
2. Hahn, F. M., and Poulter, C. D. (1995) J. Biol. Chem. 270, 11298–11303
3. Kaneda, K., Kuzuyama, T., Takagi, M., Hayakawa, Y., and Seto, H. (2001) Proc. Natl. Acad. Sci. U. S. A. 98, 932–937
4. Steinbacher, S., Kaiser, J., Gerhardt, S., Eisenreich, W., Huber, R., Bacher, A., and Rohdich, F. (2003) J. Mol. Biol. 329, 973–982
5. Carrigan, C. N., and Poulter, C. D. (2003) J. Am. Chem. Soc. 125, 9008–9009
6. Bonnanno, J. B., Edo, C., Eswar, N., Pieper, U., Romanowski, M. J., Ilvín, Y., Gerchman, S. E., Kyčia, H., Studier, F. W., Sali, A., and Burley, S. K. (2001) Proc. Natl. Acad. Sci. U. S. A. 98, 12986–12991
7. Durbecq, V., Sainz, G., Oudjama, Y., Clantin, B., Bompadre-Gilles, C., Tricot, C., Cailliet, J., Stalon, V., Droogmans, L., and Villeret, V. (2001) EMBO J. 20, 1530–1537
8. Wouters, J., Oudjama, Y., Barkley, S. J., Tricot, C., Stalon, V., Droogmans, L., and Poulter, C. D. (2003) J. Biol. Chem. 278, 11903–11908
9. Wouters, J., Oudjama, Y., Stalon, V., Droogmans, L., and Poulter, C. D. (2004) Proteins 54, 216–221
10. Wouters, J., Oudjama, Y., Ghosh, S., Stalon, V., Droogmans, L., and Oldfield, E. (2003) J. Am. Chem. Soc. 125, 3198–3199
11. Oudjama, Y., Durbecq, V., Sainz, G., Clantin, B., Tricot, C., Stalon, V., Villeret, V., and Droogmans, L. (2001) Acta Crystallogr. Sect. D Biol. Crystallogr. 57, 287–288
12. Satterwhite, D. M. (1985) Methods Enzymol. 110, 92–99
13. Otwinowski, Z., and Minor, W. (1997) Methods Enzymol. 267, 307–326
14. Sheldrick, G. M., and Schneider, T. R. (1992) J. Mol. Biol. 229, 319–343
15. Roussel, A., and Cambillau, C. (1992) Arch. Biochem. Biophys. 296, 367–377
16. Minetti, C. A. S., Tai, J. Y., Blake, M. S., Pullen, J. K., Liang, S. M., and Remeta, D. P. (2001) Proteins 41, 147–147
17. Minetti, C. A. S., Tai, J. Y., Blake, M. S., Pullen, J. K., Liang, S. M., and Remeta, D. P. (2001) Proteins 41, 158–158
18. Ragone, R., Colonna, G., Balestrieri, C., Servillo, L., and Irace, G. (1984) Biochemistry 23, 1871–1875
19. Bagger, H. L., Fuglsang, C. C., and Westh, P. (2003) Biochemistry 42, 10985–10990
20. DeLano, W. L. (2002) The PyMOL Molecular Graphics System, Version 0.99, DeLano Scientific, San Carlos, CA
21. Higgins, D., Thompson, J., and Gibson, T. J. (1994) Gene 159, 467–4680