Articles

A Double Mutation at the Tip of the Dimer Interface Loop of Triosephosphate Isomerase Generates Active Monomers with Reduced Stability†

Wolfgang Schliebs,‡,§ Narmada Thanki,‡ Rainer Jaenicke,|| and Rik K. Wierenga*,‡

EMBL, Meyerhofstrasse 1, D69126 Heidelberg, Germany, and Institut für Biophysik und physikalische Biochemie, Universität Regensburg, D93040 Regensburg, Germany

Received December 16, 1996; Revised Manuscript Received April 24, 1997⊗

ABSTRACT: Triosephosphate isomerase (TIM) is a very stable dimer. In order to understand better the importance of dimerization for stability and catalytic activity, we have constructed a monomeric double-mutation variant. The dimer interface residues Thr75 and Gly76, which are at the tip of loop 3, have been substituted by an arginine and a glutamate, respectively. In wild type TIM, these two residues are at a distance of 27 Å from the active site (as measured within the same subunit). This new variant, RE-TIM, was expressed in *Escherichia coli*, purified to homogeneity, and biochemically characterized. Sedimentation equilibrium ultracentrifugation runs showed that RE-TIM is a monomer in solution. Far-UV CD spectra indicate that this new variant is folded properly and that the secondary structure contents of RE-TIM are similar to those of wild type TIM. The monomeric RE-TIM has residual TIM activity. The thermal stability of RE-TIM is lower than that for wild type TIM. CD melting curves for RE-TIM and wild type TIM show T_m values of 52 and 57 °C, respectively, in the presence of the active site ligand 2-phosphoglycolate at 1 mM. Previously, we have characterized two other monomeric forms of TIM: monoTIM and H47N-TIM. The properties of RE-TIM, H47N-TIM, and monoTIM are compared, and it is argued that the properties of RE-TIM will be very similar to those of wild type monomeric subunits. This implies that wild type monomeric subunits have some stability and are catalytically active. It is also inferred that these monomeric subunits have flexible loops which rigidify at the dimer interface on dimerization, causing a 1000-fold increase of k_{cat} and a 10-fold decrease of K_m.

Oligomerization is an important property of many proteins (Goodsell & Olson, 1993; Janin et al., 1988). It provides an additional level of complexity which can result in additional properties such as cooperativity or more complex active sites. In this paper, we focus on the monomer/dimer properties of the dimeric glycolytic enzyme triosephosphate isomerase (TIM,[1] EC 5.3.1.1.). TIM catalyzes the interconversion of dihydroxyacetone phosphate (DHAP) and D-glyceraldehyde 3-phosphate (GAP) (Knowles, 1991).

TIM is a very stable dimer [the estimated K_d is smaller than 10^{-11} M (Borchert et al., 1994)], consisting of two identical subunits. The triosephosphate isomerase subunit has the $(\beta/\alpha)_8$ fold with a central core of eight parallel β-strands (strands 1−8) covered on the outside by eight α-helices (helices 1−8). The C-terminal end of each strand is connected to the subsequent helix by a loop, denoted by the number of the preceding β-strand. These loops are the major constituents of the active site. Loops 1−4 are additionally involved in the formation of the interface between the two subunits. Loop 3 protrudes out of the bulk of its own subunit. Loop 3 of subunit 1 docks into a deep pocket between loop 1 and loop 4 of subunit 2. It is in van der Waals contact and hydrogen bonding contact with active site residues Lys13 (loop 1) and Glu97 (loop 4) of the other subunit (Schliebs et al., 1996). The active site pocket is therefore close to the dimer interface, near to the docked loop 3 of the other subunit. However, the residues involved in binding the substrate and participating in catalysis all belong to the same subunit. The active site pocket is a deep pocket, as shown in Figure 1.

There are numerous studies of monomer/dimer properties of dimeric proteins. In general, there are tight interactions between the subunits of a dimeric protein, which implies that the monomers by themselves are less stable and therefore difficult to study (Jaenicke, 1987, 1996). In agreement with this, a systematic analysis of the crystal structures of dimeric proteins has shown the existence of very extensive interactions across the dimer interface with good shape complementarity (Jones & Thornten, 1995). Investigations into the folding/association pathways of dimeric proteins have shown that in some cases structured monomeric intermediates can be observed, for example for malate dehydrogenase and TIM (Jaenicke, 1987). Denaturation studies with other dimeric proteins, for example glutathione *S*-transferase (Erhardt &

† This research was supported by EU Grants BIOT-CT90-0182, CHRX-CT93-0173, and ERBCHGE-CT94-0062.

* To whom correspondence should be addressed.

‡ EMBL.

§ Present address: Institut für Physiologische Chemie, Ruhr Universität, D44780 Bochum, Germany.

|| Universität Regensburg.

⊗ Abstract published in *Advance ACS Abstracts,* August 1, 1997.

[1] Abbreviations: BPMC, biased probability Monte Carlo; DHAP, dihydroxyacetone phosphate; DTT, reduced dithiothreitol; EDTA, ethylenediaminetetraacetic acid; GAP, D-glyceraldehyde 3-phosphate; MOPS, 3-(*N*-morpholino)propanesulfonic acid; PGH, phosphoglycolohydroxamate; TIM, triosephosphate isomerase; 2PG, 2-phosphoglycolate.

S0006-2960(96)03086-3 CCC: $14.00

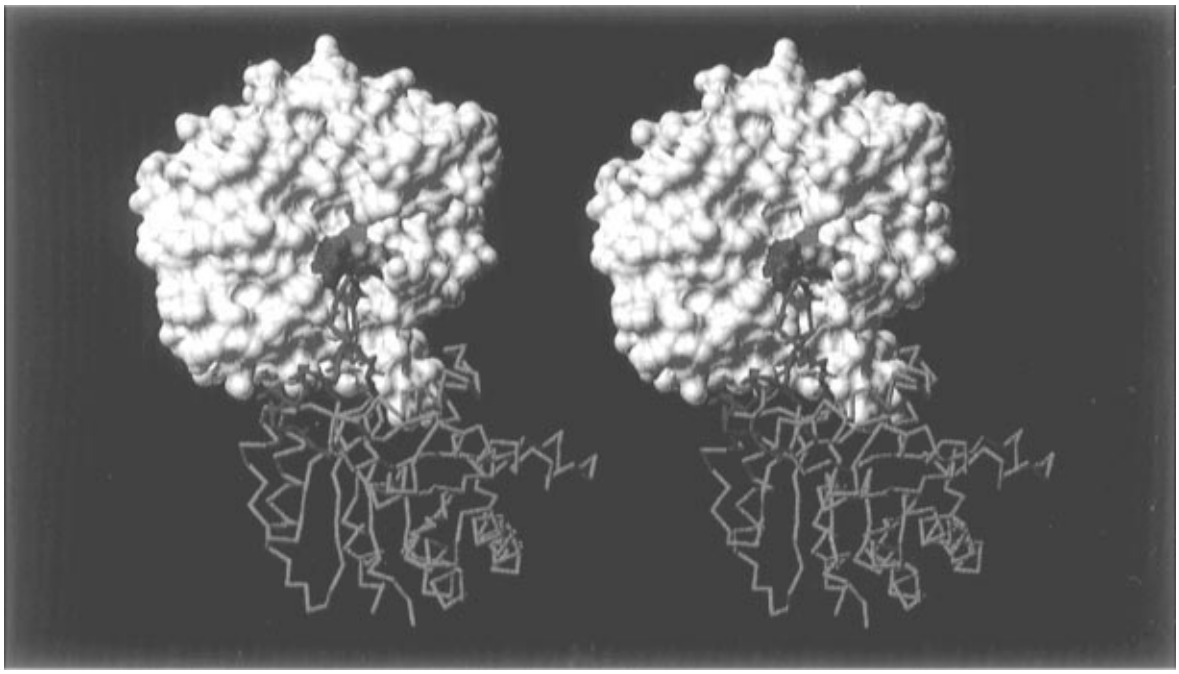

FIGURE 1: Stereoview into the active site pocket of the TIM dimer. Loop 3 of the lower subunit contacts Lys13 (blue) and Glu97 (gray) of the upper subunit. Further into this pocket are His95 (red) and Glu167 (green). The N terminus of the lower subunit is at the first β-strand; the C terminus is after the last α-helix.

Dirr, 1995) or bacterophage λ cro (Mossing & Sauer, 1990), have shown that the unfolding can be a cooperative and concerted two-state transition between the native dimer and the unfolded monomeric protein, with no evidence for a stable monomeric intermediate. Sometimes the denaturation conditions determine the unfolding/refolding mechanism; for example, in ROP, a strict two-state behavior is seen during thermal unfolding of the dimer, whereas in the presence of 2.5 M guanidine hydrochloride, ROP dissociates first into monomers at elevated temperatures before it denatures (Steif et al., 1993). For this protein, the relative stability of the monomer by itself and the monomer in the context of the dimer could be quantified. It was found that the extrapolated value of ΔG for unfolding at 19 °C, in the absence of guanidine hydrochloride, equals 22.4 kJ (mol of monomer)$^{-1}$ for the monomer by itself and 35.9 kJ (mol of monomer)$^{-1}$ in the context of the dimer (Steif et al., 1993). The examples discussed above show that the stability of the monomeric protein can vary from completely unstable (such as that for cro) to significantly stable (such as that for ROP).

Concerning TIM, several attempts have been made to study the properties of the monomeric wild type subunits by using various techniques such as (i) studying the refolding kinetics of the denatured protein (Zabori et al., 1980), (ii) measuring the activity of monomers cross-linked to a solid matrix (Fell & Branford White, 1975), or (iii) studying the effect of pressure-induced monomerization (Rietveld & Ferreira, 1996). None of these approaches has given much insight into the catalytic properties of the monomeric subunits of wild type TIM.

In some cases, well-designed loop transplantations have resulted in stable monomeric variants of dimeric proteins; examples are monomers of trypanosomal TIM (Borchert et al., 1993a), bacteriophage λ cro (Mossing & Sauer, 1990), and interleukin-5 (Dickason & Huston, 1996). In these studies, rather large sequence changes have been introduced in the wild type protein; therefore, to what extent the properties of these monomers reflect the properties of the wild type monomeric subunits is not clear. Site-directed mutagenesis of carefully selected interface residues seems to be the best way to obtain information concerning the properties of monomeric wild type subunits. These studies have shown that such monomers can be equally active [aldolase (Beernink & Tolan, 1996)], less active [for example, superoxide dismutase (Bertini et al., 1994) or malate dehydrogenase (Breiter et al., 1994)], or completely inactive [such as tyrosyl-tRNA synthetase (Ward et al., 1987) or prostatic acid phosphatase (Povari et al., 1994)]. Crystal structures have not yet been reported for any of these monomeric proteins, but crystallographic studies of monomeric malate dehydrogenase have been initiated (Breiter et al., 1994).

Point mutation variants of TIM with considerably less stable dimers have been made. However, these variants have not given much insight into the catalytic properties of the wild type TIM subunits because these variants either have been incompletely characterized [yeast TIM (Casal et al., 1987)] or are not suitable analogues of the wild type TIM subunit (human TIM and trypanosomal TIM). The monomeric variant of human TIM, which turned out to be inactive, was constructed by making two point mutations: M14Q (in loop 1) and R98Q (in loop 4) (Mainfroid et al., 1996). Other experiments have shown that the R98Q single-point mutation variant is also inactive, whereas the M14Q variant is still active, although both variants can still form dimers (Mainfroid et al., 1996). Why is the R98Q variant of human TIM inactive? This can be rationalized as follows. (i) Arg98 is a completely conserved residue, close to the active site pocket. (ii) Arg98 stabilizes the helical fragment (residues 95−100) of loop 4 via a salt bridge with the side chain of Glu104. This helical fragment starts off with His95 which is a catalytic residue. The importance of this Arg98−Glu104

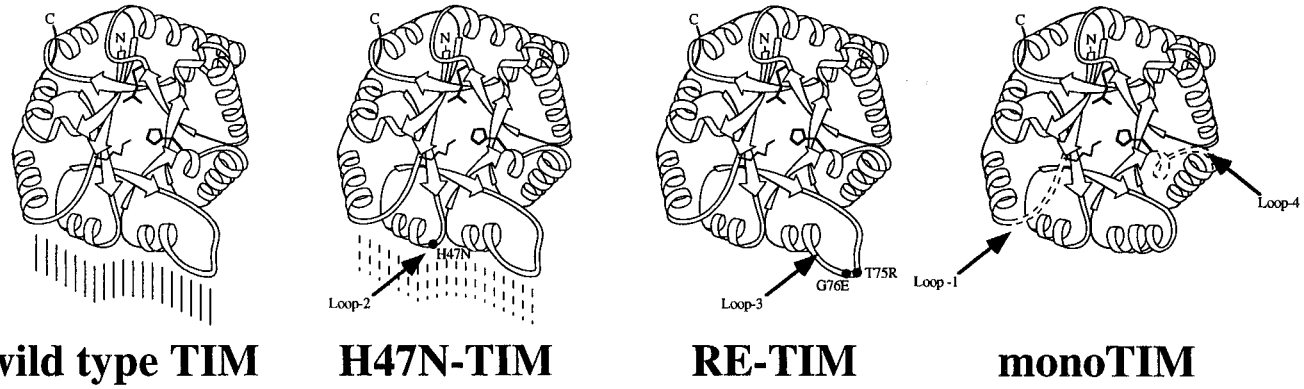

FIGURE 2: Schematic overview of the four different TIM variants whose properties have been studied. The positions of the side chains of the catalytic residues Lys13 (loop 1), His95 (loop 4), and Glu167 (loop 6) are drawn. N and C identify the N terminus and the C terminus, respectively. The point mutations of H47N-TIM (in loop 2) and RE-TIM (in loop 3) are shown. The approximate positions of the dimer interfaces of wild type TIM (tight interface, continuous lines) and H47N-TIM (weak interface, dotted lines) are indicated. In the monoTIM picture, loop 1 and loop 4 are drawn as dotted lines to indicate the conformational flexibility.

Table 1: Kinetic Parameters of Wild Type TIM, MonoTIM, and RE-TIM

	wild type TIM	monoTIM[b]	RE-TIM
k_{cat} (GAP) (min^{-1})	3.7×10^5 [a]	3.1×10^2	1.3×10^2
K_m (GAP) (mM)	0.25 ± 0.05 [a]	4.1 ± 0.6	1.4 ± 0.2
k_{cat}/K_m (GAP) (min^{-1} mM^{-1})	1.5×10^6	75	93
k_{cat} (DHAP) (min^{-1})	6.5×10^4 [a]	12	28
K_m (DHAP) (mM)	1.2 ± 0.1[a]	12.2 ± 1.9	17.2 ± 5.8
k_{cat}/K_m (DHAP) (min^{-1} mM^{-1})	5.4×10^4	1.0	1.6
K_i (PGH) (mM)	0.008 ± 0.001[b]	0.50 ± 0.07	1.75 ± 0.3
K_i (2PG) (mM)	0.026 ± 0.007[b]	0.052 ± 0.007	0.034 ± 0.005

[a] Lambeir et al. (1993). [b] Schliebs et al. (1996).

salt bridge is emphasized by two observations. (i) In each of the four monoTIM structures, this salt bridge is present, including the monoTIM structure where the helical fragment of loop 4 has undergone a major shift away from the active site, due to φ and ψ changes of Gly94 and His95 (Borchert et al., 1993a). (ii) Glu104 and Arg98 are completely conserved in 45 TIM sequences, and the human TIM variant E104D has been found to be a genetic disorder causing severe TIM deficiency leading to death within the first 6 years (Pekrun et al., 1995). In the absence of this salt bridge (such as in the R98Q variant), the helical fragment might just be unstable and might just adopt a random coil conformation, blocking the active site or at least placing the His95 side chain in a position that is incompatible with catalysis.

Previously, we have characterized two different monomeric forms of trypanosomal TIM: H47N-TIM and monoTIM (Figure 2). H47N-TIM (Borchert et al., 1993b, 1995) is constructed by changing His47 into an asparagine. In the dimer, His47 is buried at the dimer interface, interacting, via a water molecule, with a buried aspartate side chain of the other subunit. It could be shown that at low protein concentrations H47N-TIM is a compactly folded, monomeric protein, less stable than the wild type dimer (Borchert et al., 1994). MonoTIM has been engineered from wild type TIM by changing the 15-residue dimer interface loop 3 into an 8-residue loop. MonoTIM is a monomeric protein (Borchert et al., 1994). Several crystal structures of monoTIM have been determined. It was found that loop 1 (with the catalytic Lys13) was disordered in each liganded structure. Loop 4 (with the catalytic His95) is well ordered in each structure but shows conformational heterogeneity. In three structures (Schliebs et al., 1996), it adopts the same conformation as seen in wild type, but in one structure (Borchert et al., 1993a) the φ and ψ values of Gly94 and His95 are changed so they cause the helical fragment of loop 4, including His95, to adopt a swing-out position. In wild type TIM, loop 1 and loop 4 are very rigid.

The monomeric TIM variants H47N-TIM and monoTIM are not ideal analogues of the monomeric wild type subunit. H47N-TIM is only a monomer at low protein concentrations; at 3 mg/mL, approximately 50% of the protein is monomer, suggesting a K_d of 5×10^{-5} M. Since the dimeric form of H47N-TIM is very active, it is impossible to measure reliably the residual catalytic activity of the monomer. MonoTIM is a true monomer. However, one cannot, *a priori*, be sure that its properties will be the same as those for the monomeric wild type subunit, because monoTIM was constructed by shortening the interface loop (loop 3). Nevertheless, it is reasonable to expect that the active site properties of monoTIM would be similar to those of the monomeric wild type TIM subunit; after all, the loop 3 residues are far away from the active site of its own subunit. For example, the beginning and end of loop 3 are approximately 17 Å away from the active site, whereas the tip of loop 3 is even at a distance of 27 Å. MonoTIM has residual catalytic activity (Table 1); it is approximately 1000-fold less active than wild type. Its much reduced activity is probably due to the mobility of the two catalytic loops, but there will be other contributing factors as well, such as the much more solvent accessible active site in monoTIM.

Here, we describe the properties of another monomeric form of trypanosomal TIM, which was made by making two, carefully selected, point mutations at the tip of the dimer interface loop 3. This variant, in which loop 3 has the same length as in wild type TIM, is referred to as RE-TIM, and it is shown that RE-TIM is indeed a monomer with

residual catalytic activity and stability. Its properties are compared with those of H47N-TIM and monoTIM, and it is argued that RE-TIM is a good analogue of the wild type monomeric subunit. One of the important implications is that the wild type TIM subunit also has residual catalytic activity.

MATERIALS AND METHODS

Materials. DNA oligonucleotides were synthesized by the EMBL oligonucleotide synthesis service. GAP, DHAP, and 2-phosphoglycolate (2PG) were purchased from Sigma. Phosphoglycolohydroxamate (PGH) was synthesized by R. Eritja (EMBL) as described earlier (Schliebs et al., 1996). All other reagents were of analytical grade.

DNA Manipulations. Site-directed mutagenesis was carried out by PCR using the overlap-extension method (Higuchi, 1990). Two variants were made. In the first variant, Thr75 was replaced by an arginine. For the construction of the T75R variant, the plasmid pet3aTIM (Borchert et al., 1994) was used as a template. The sequences of the mutagenic primers were 5′-TT*C**CG**CG*GCGAAGTCTC-CCTGCC-3′ (sense) and 5′-GGGAGACTTCG*CC**G**CG*-*G*AAGGCACCGCTCTT-3′ (antisense), respectively. Mismatched bases are shown in bold; the changed codon is underlined, and a new *Sac*II site which was generated to facilitate the identification of correct clones is indicated in italics. To introduce a second mutation at the position of Gly76 into the same protein PCR was performed on the (T75R)pet3aTIM-DNA. The sequences of the synthesized oligonucleotides used as mutagenic primers are 5′-TGCCT-TCCGCG**AA**GAAGTCTCCCT-3′ (sense) and 5′-AGG-GAGACTTC**TT**CGCGGAAGGCA-3′ (antisense). The amplified DNA fragments carrying T75R were subcloned into pET3aTIM. The fragments with the additional G76E substitution were subcloned into pET3aTIM(T75R), allowing an easier identification due to the loss of the *Sac*II site. The plasmid DNA was then isolated from *Escherichia coli* strain XL1-Blue (Bullock et al., 1987) for restriction site analysis. The correct sequence for both TIM variants has been confirmed by DNA double-strand sequencing using the USB sequence kit.

Protein Expression and Purification. The expression of all TIM variants was carried out in *E. coli* strain BL21(DE3). Growth conditions and purification procedures for wtTIM, H47N-TIM, and monoTIM have been described previously (Borchert et al., 1993b, 1994). Since most of the RE-TIM was insoluble under these conditions, we lowered the expression rate to background expression. The low expression rate was achieved simply by not adding the inducer isopropyl 1-thio-β-D-galactoside. The RE-TIM-expressing *E. coli* strain was grown in 1 L cultures of M9 medium supplemented with 100 μg/mL ampicillin at 30 °C until the cells reached high density. Approximately 5 mg of pure protein could be obtained from 1 L of culture with the following purification protocol. Cells were lysed in two passages with a French press in 100 mM triethanolamine (TEA, pH 8.0), 1 mM phenylmethanesulfonyl fluoride, and 200 mM NaCl. The cell lysate was centrifuged at 44000*g* for 30 min. The supernatant was diluted 8-fold with 25 mM TEA (pH 8.0) and 1 mM PMSF and loaded onto a SP/Sepharose column equilibriated with 25 mM TEA (pH 8.0) and 25 mM NaCl. Protein was eluted with a 25 to 125 mM NaCl gradient. All solutions used for lysis and purification were supplemented with 5 mM DTT, 1 mM EDTA, and 1 mM sodium azide. The purity of all monoTIM variants was checked by SDS/PAGE analysis, using Coomassie staining. Protein concentrations were determined with Bradford reagent using bovine serum albumin as a standard.

Enzyme Assays. Standard assays for the conversion of the triosephosphates in both directions and determination of kinetic parameters were carried out at 25 °C as described previously (Schliebs et al., 1996). One activity unit (u) represents the conversion of 1 μmol of substrate per minute. For the calculation of kinetic parameters and error estimates, the programs Ultrafit (Biosoft) and Grafit (Sigma) were used. The enzyme-catalyzed formation of methylglyoxal was tested under standard assay conditions with 2 mM GAP and various amounts of RE-TIM. The assay buffer also contained glyoxylase I and glutathione. The conversion of methylglyoxal into *S*-lactoylglutathione was followed spectrophotometrically at 240 nm (Gawehn & Bergmeyer, 1974).

CD Measurements. CD spectra at different temperatures were recorded on a Jasco J-710 spectropolarimeter. For far-UV spectra, 0.2 cm path length cuvettes were used. The protein (0.2 mg/mL) was dissolved in 20 mM MOPS buffer (pH 7.0) containing also 1 mM EDTA and 1 mM azide. Temperature denaturation studies in the presence of 1 mM 2PG were carried out by monitoring the ellipticity at 222 nm at increasing temperatures from 20 to 70 °C. The temperature was increased by using a heating rate of 20 °C/h. The protein solution (0.2 mg/mL) was in each case a 20 mM MOPS buffer (pH 7.0) containing also 1 mM DTT, 1 mM EDTA, 1 mM azide, and 1 mM 2PG. Denaturation was found to be irreversible for all samples.

Sedimentation Analysis. Experiments were performed at room temperature and at 4 °C making use of Beckman Spinco Model E and XLA analytical ultracentrifuges (12 mm double-sector and six-hole short-column cells with Sapphire windows; scanning wavelengths of 235 and 280 nm). Prior to the experiments, the enzyme solutions were subjected to equilibrium dialysis in 20 mM TEA-HCl buffer (pH 8.0) containing 200 mM NaCl, 5 mM DTT, 1 mM EDTA, and 1 mM sodium azide.

Sedimentation velocity experiments at 0.08−0.23 mg/mL enzyme concentrations were performed at 44 000 and 30 000 revolutions/min; sedimentation coefficients were corrected for 20 °C and water viscosity ($\upsilon_p = 0.735$ mL/g, $\rho_o = 1.009$ g/mL). High-speed sedimentation equilibria according to Yphantis (1964) were performed at 16 000 and 30 000 revolutions/min. Evaluation was based on computer programs provided by G. Böhm (University of Regensburg), as well as by D. Schubert and P. Schuck (University of Frankfurt), the first based on the linearization in a ln c vs r^2 plot and the second on a fitting program using $M = 26\,850$ as monomer mass.

Modeling and Structural Analysis. ICM (Abagyan et al., 1994), WHAT IF (Vriend, 1990), and O (Jones et al., 1991) were used for modeling and structural analysis. For the loop 3 modeling, the biased probability Monte Carlo (BPMC) calculations were done as implemented in ICM (Abagyan & Totrov, 1994) and as described before (Thanki et al., 1997). For the calculations, the wild type TIM structure (5TIM in the PDB) was used, after removing all non-protein atoms and after regularization. In these calculations, the

dihedrals of the loop 3 residues of both subunits were unfixed. Also, the dihedrals of neighboring side chains (within a 6 Å sphere of the loop 3 regions) were unfixed.

RESULTS

Construction of a Monomeric Variant. In dimeric TIM, most of the dimer interface contacts are made between atoms of loop 3 of one subunit and atoms lining a deep groove of the other subunit. The sequence at the tip of loop 3 is FT-(75)G(76)E. The sequence FTGE is alomost completely conserved in 45 TIM sequences. The F position is sometimes a trypophan (two times) and sometimes a tyrosine (seven times). The TG sequence is completely conserved. The E position is sometimes an aspartate (three times) or a threonine (one time). Gly76 has φ and ψ values (90° and −15°) not allowed for non-glycine residues. The side chain of Thr75 is pointing into the active site pocket of the other subunit, and it is hydrogen bonded to the side chains of the active site residues Asn11 (loop 1) and Glu97 (loop 4) of the other subunit. Residues at the tip of loop 3 were selected for making amino acid substitutions to destabilize the dimer. They were chosen for the following reasons. (i) These residues make many contacts with the other subunit, and (ii) these residues are far away from the active site of their own subunit.

In a first attempt, Thr75 was substituted with a larger, charged amino acid (arginine), to induce clashes in the loop 3 binding pocket. This variant is referred to as R-TIM. With the BPMC simulation protocol of ICM (unfixing the dihedrals of loop 3 residues 73−77), only one low-energy conformation was found. In this low-energy conformation, all φ and ψ values of the unfixed loop 3 region of both subunits are in the allowed region of the Ramachandran plot. The arginine side chain fills the bottom of the active site pocket, but its calculated positon does not overlap with the actual ligand binding site. However, two close contacts (shorter than 3 Å) are predicted between non-hydrogen bonding atoms. From this modeling exercise, it was anticipated that R-TIM would be a monomer, because of the buried charge of the arginine side chain, as well as because of the nonbonded contacts that were too close. R-TIM was made, expressed in *E. coli*, and purified to homogeneity. Surprisingly, the characterization suggested that active R-TIM dimers are in equilibrium with less active monomers. For example, when the reaction assay is started by diluting a 2.5 mg/mL R-TIM stock solution into the standard assay buffer (final concentration in the cuvette is 10 μg/mL), the initial activity is 16 u/mg, but after 30 min, it is decreased to 1 u/mg. A similar activity is measured when the assay is started in a solution of 25 μg/mL R-TIM in assay buffer. Apparently, the dimeric active site pocket can accommodate the presence of this bulky and charged side chain, without completely losing its catalytic activity. This was an unexpected result, given also the observation of the strict evolutionary conservation of Thr75.

Subsequently, the variant (T75R/G76E) referred to as RE-TIM was considered. First, modeling calculations were done with this variant. The modeled R-TIM structure was taken as the starting model. For these calculations, the dihedrals of residues 73−78 of both subunits were unfixed. Again, the BPMC run found only one low-energy conformation. In this low-energy conformation, all the new charged side chains are pointing inward. There are nine close contacts (shorter than 3 Å) between nonbonded atoms. In addition, there are two outliers in the Ramachandran plot for the calculated loop 3 conformations. Apparently, in this variant, these predicted unfavorable interactions are sufficient to prevent dimerization because, as is explained in the further sections, this variant is indeed a monomer.

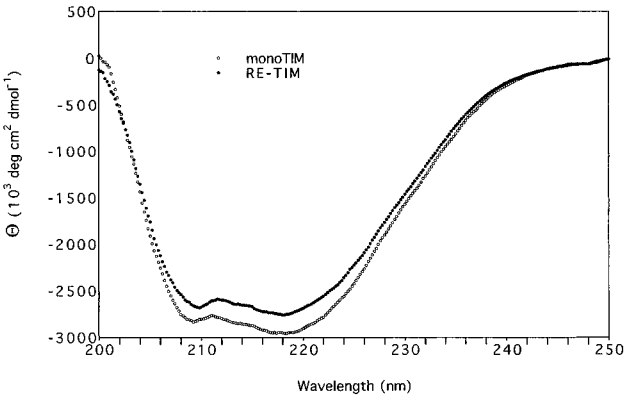

FIGURE 3: Far-UV spectra of RE-TIM (●) and monoTIM (○). The molar ellipticity is plotted.

Purification and Biophysical Properties of RE-TIM. The expression and purification of RE-TIM required some modification with respect to the standard protocols for expression and purification of previous monomeric TIM variants. This is described in Materials and Methods. These modifications were caused by the lower stability and lower solubility of RE-TIM. With the modified protocol pure RE-TIM is obtained, which is soluble at 5 mg/mL in a 25 mM TEA buffer (pH 8.0) with 1 mM EDTA, 1 mM sodium azide, 1 mM DTT, and 100 mM NaCl. Figure 3 shows the far-UV CD spectra of RE-TIM and monoTIM. Apart from scaling differences, due to small differences in protein concentration, these spectra are virtually identical, showing that the secondary structure contents of RE-TIM and monoTIM are similar. The sedimentation equilibrium runs of a solution of RE-TIM showed no indication for dimer formation, even at the bottom of the cell, i.e. at concentrations of up to 1.2 mg/mL. There is a perfect fit of the concentration profiles, assuming exclusively the monomer (M = 26 850) and that no dimer is present. The sedimentation coefficient $S_{20,w}$ = 2.52 S.

Kinetic Properties of RE-TIM. RE-TIM has residual TIM activity. To exclude the possibility that this activity is due to the formation of small amounts of a more active dimeric form, the protein concentration dependence of the specific activity (units per milligram) was measured. No concentration dependence was found. For example, when the reaction assay is started by diluting a 3.5 mg/mL RE-TIM stock solution into the standard assay buffer (final concentration in the cuvette is 10 μg/mL), the initial activity is 1.5 u/mg, and during 30 min, the activity remains constant. Also, when the assay is started in a solution of 10 μg/mL RE-TIM in assay buffer, the activity is 1.5 u/mg.

A comparison of kinetic data of RE-TIM, monoTIM, and wtTIM is shown in Table 1. The steady state kinetics of RE-TIM were determined for both the forward reaction (with GAP) and the reverse reaction (with DHAP). k_{cat} for both directions is approximately 2500-fold lower compared with that of dimeric wild type TIM. The K_m values for GAP and DHAP are 6 and 14 times higher, respectively, than that of

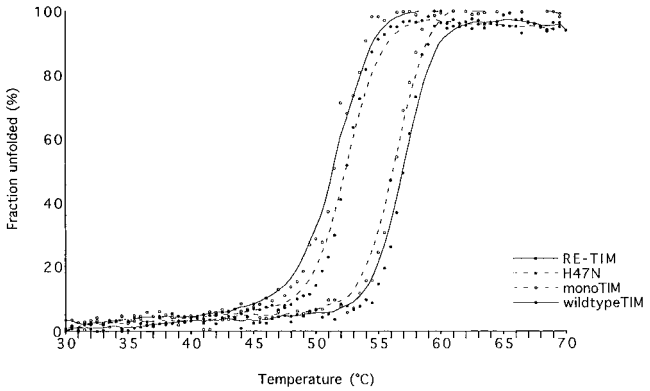

FIGURE 4: CD melting curves of RE-TIM, H47N-TIM, monoTIM, and wild type TIM. The fraction of unfolded protein was obtained by measuring the CD signal at 222 nm at increasing temperatures, assigning the 0 and 100% values to the pre- and post-transition baselines, respectively: RE-TIM (○, continuous), H47N-TIM (●, dotted), monoTIM (○, dotted), and wild type TIM (●, continuous).

wild type. The kinetic data of RE-TIM are strikingly similar to the kinetic parameters obtained for monoTIM. Additionally, the second-order rate constants (k_{cat}/K_m) (indicating specificity) are almost identical for RE-TIM and monoTIM. The K_i values for the substrate analogue 2PG and the transition state analogue PGH have also been determined. Whereas the affinity of 2PG is not affected in RE-TIM and monoTIM, the K_i for PGH is nearly 200 times higher for RE-TIM and monoTIM, compared to that for wild type TIM. No RE-TIM-catalyzed formation of methylglyoxal could be detected in the methylglyoxal synthase assay.

Stability of RE-TIM. The thermal stability of RE-TIM and H47N-TIM has been studied by temperature-dependent CD measurments at a wavelength of 222 nm. The protein concentration in both cases was 0.2 mg/mL. It has been shown before that H47N-TIM is predominantly monomeric at this concentration (Borchert et al., 1995). The CD melting curves of RE-TIM and H47N-TIM as well as those for monoTIM and wild type TIM are shown in Figure 4. For the experiments illustrated in Figure 4, 1 mM 2PG was added to the protein solutions. The thermal stability of the H47N-TIM monomer and the RE-TIM monomer is virtually the same; for both proteins, the T_m value is approximately 52 °C, whereas the T_m value of wild type TIM is 57 °C. The T_m value for monoTIM is also approximately 57 °C.

DISCUSSION

RE-TIM is a double-mutation variant of trypansomal TIM, which is a dimeric enzyme. The two point mutations are at the tip of the long dimer interface loop 3, which in wild type TIM fits precisely in a pocket close to the active site of the other subunit. The mutations have been chosen (by modeling studies) to prevent dimerization. The biochemical characterization of RE-TIM has shown that RE-TIM has residual activity as well as stability. The far-UV CD measurements (Figure 3) of RE-TIM indicate that the secondary structure contents of RE-TIM and monoTIM are similar, in agreement with the notion that RE-TIM has the same fold as monoTIM. As the crystal structure of monoTIM is known, this implies that RE-TIM has the same fold as wild type TIM.

Previously reported experiments with H47N-TIM (Borchert et al., 1993b), as well as the measurements with R-TIM (reported here), indicate that these two variants can form dimers with higher specific activity at higher protein concentrations. On dilution of the concentrated protein solutions of these two variants in the assay buffer, the specific activity decreases in time due to the dissociation of the active dimer into a less active monomer. Ultracentrifugation studies showed that the lower specific activity of RE-TIM is not due to a possibly inactivated dimer because these studies showed that, even at 1.2 mg/mL, no dimer formation could be detected. To exclude the possibility of the formation of small amounts of more active dimers, the protein concentration dependence of the specific activity was also measured. It was found that in the activity assays the specific activity is completely independent of the protein concentration. It is therefore concluded that RE-TIM dimerization is not induced at higher protein concentrations or in the presence of substrate or substrate analogues.

RE-TIM is an artificial protein with disordered loops. One of these loops, loop 3, is 15 residues long. In wild type TIM, this loop protrudes 13 Å out of the bulk of the subunit. The sequence of this loop in wild type TIM is IAKSGAF**T-G**EVSLPI. In RE-TIM, the TG sequence has been replaced by RE. The TG sequence, at the tip of the loop, is completely conserved in 45 TIM sequences, allowing for an optimal fit, including many stabilizing interactions, in the loop 3 binding pocket of the other subunit of the dimer. In the absence of this binding pocket, as in RE-TIM, this loop points into the solvent. Apparently, this is a very unfavorable conformation, as indicated by a lower than wild type stability and low solubility as well as by the difficulties in expressing RE-TIM. Interestingly, RE-TIM has the same stability as the monomeric form of H47N-TIM (Figure 4), which is lower than the stability of the other monomeric TIM, monoTIM, in which loop 3 has been replaced by a much shorter loop. The similar stability of H47N-TIM and RE-TIM agrees very well with the assumption that the larger loop 3, present in both H47N-TIM and RE-TIM, destabilizes the protein.

The monomeric RE-TIM has two more important properties which need to be emphasized and which need to be compared with wild type TIM and monoTIM. (i) RE-TIM is less active than wild type TIM but as active as monoTIM, and (ii) the affinity of RE-TIM and monoTIM for the transition state analogue PGH is the same. This affinity is much lower than that observed for wild type TIM (Table 1). Apparently, the dimerization changes the active site pocket in such a way that its complementarity to PGH is improved. PGH is a transition state analogue. Therefore, the lower affinity of RE-TIM and monoTIM for PGH and the lower catalytic activity of RE-TIM and monoTIM agree very well with the notion that the optimal active site, such as is seen in wild type TIM, is also optimally complementary to a good transition state analogue.

Monomeric RE-TIM is generated by point mutations of two conserved residues at the tip of loop 3, whereas monomeric monoTIM was obtained by replacing the protruding loop 3 with a much shorter segment. This deletion is near the active site; therefore, as discussed in the introductory section, the deletion variant cannot, *a priori*, be considered a good analogue of the wild type subunit. However, the RE-TIM variant is a good analogue of the wild type monomeric subunit. Since RE-TIM has residual activity, this also implies that the monomeric wild type subunit has residual activity. The kinetic properties of RE-TIM and monoTIM are virtually identical (Table 1). This implies that the

FIGURE 5: Two loop 4 conformations. The black Cα trace is for wild type TIM, and S2 and Q250 label the N terminus and C terminus, respectively. The side chains of the catalytic residues Lys13, His95, and Glu167 are shown in blue. His95 marks the beginning of loop 4. The swing-out conformation of loop 4, as seen in one of the monoTIM structures, is colored red. This swing-out conformation is caused by two different main chain dihedrals: φ (Gly94) and ψ (His95), which have both changed by approximately 180° (Borchert et al., 1993a). The green Cα trace is the dimer interface loop 3 of the other subunit.

active site properties of monoTIM are the same as those for RE-TIM and therefore for the monomeric wild type subunit as well. The crystallographic studies of monoTIM have shown that in monoTIM the two catalytic loops, loop 1 and loop 4, are mobile in solution. Nevertheless, mutagenic studies have shown that Lys13 (in loop 1) and His95 (in loop 4) are essential for catalysis, supporting the hypothesis that in the presence of substrate loop 1 and loop 4 are induced to adopt a conformation suitable for catalysis (Schliebs et al., 1996). It also seems therefore that in the monomeric wild type subunit loop 1 and loop 4 are disordered. On dimerization, the disordered loop 1, loop 3, and loop 4 interact with each other and after successful assembly form a very rigid dimer interface. The assembly of the two subunits is therefore an induced fit process. The last step of this induced fit mechanism could be the movement of loop 4 from the swing-out position, as observed in one of the monoTIM structures (Borchert et al., 1993a), to the swing-in position, as observed in the other monoTIM structures (Schliebs et al., 1996) and in wild type TIM. This loop 4 movement is illustrated in Figure 5. This proposed induced fit mechanism for the dimerization is therefore not the docking of two complementary surfaces, such as for example that observed in the complex formation between trypsin and trypsin inhibitor (Bode & Huber, 1991) or between antibody and antigen (Chothia, 1991). For these examples of protein−protein recognition, two stable proteins with surfaces of complementary shape recognize each other. The resulting protein complexes are very stable, with dissociation constants in the range of $10^{-8}-10^{-13}$ M (Janin & Chothia, 1990). It has been pointed out that the surfaces buried on formation of these protein−inhibitor complexes is approximately 800 Å^2 per participating protein (Janin & Chothia, 1990; Janin, 1995); this is much less compared with the formation of TIM dimers, where each subunit buries approximately 1300 Å^2 on formation of the dimer (Wierenga et al., 1992). The larger buried surface area provides for additional favorable interactions which compensate for the extra loss of entropy due to the rigidification of the flexible loops.

The deduced mechanism of dimerization of TIM is in good agreement with the consecutive folding−association mechanism, as predicted from folding studies (Zabori et al., 1980). First, the monomers fold into structured monomers, and these structured monomers are able to specifically recognize each other and subsequently undergo further reshuffling, leading to the final tertiary and quaternary structure. The dimerization induces strain in loop 1 of the dimeric molecule. In particular, the φ and ψ values of the catalytic residue Lys13 are in the disallowed region of the Ramachandran plot, and also, the χ_2 of Trp12 is close to zero. Apparently, this strain, induced by dimerization, is required for the creation of a deep and rigid active site pocket with an optimal shape for efficient catalysis.

ACKNOWLEDGMENT

It is a great pleasure to acknowledge that this research emerged from a project initiated by Profs. Hol (Seattle) and Opperdoes (Brussels). We also gratefully acknowledge stimulating discussions with Dr. Borchert (Copenhagen). The XLA ultracentrifugation experiments were kindly performed by Prof. Schubert (Frankfurt).

REFERENCES

Abagyan, R. A., & Totrov, M. M. (1994) *J. Mol. Biol. 235*, 983−1002.

Abagyan, R. A., Totrov, M., & Kuznetsov, D. (1994) *J. Comput. Chem. 15*, 488−506.

Beernink, P. T., & Tolan, D. R. (1996) *Proc. Natl. Acad. Sci. U.S.A. 93*, 5374−5379.

Bertini, I., Piccoli, M., Viezzoli, M. S., Chiu, C. Y., & Mullenbach, G. T. (1994) *Eur. Biophys. J. 23*, 167−176.

Bode, W., & Huber, R. (1991) *Curr. Opin. Struct. Biol. 1*, 45−52.

Borchert, T. V., Abagyan, R., Radha Kishan, K. V., Zeelen, J. P., & Wierenga, R. K. (1993a) *Structure 1*, 205−213.

Borchert, T. V., Pratt, K., Zeelen, J. P., Callens, M., Noble, M. E. M., Opperdoes, F. R., Michels, P. A. M., & Wierenga, R. K. (1993b) *Eur. J. Biochem. 211*, 703−710.

Borchert, T. V., Abagyan, R., Jaenicke, R., & Wierenga, R. K. (1994) *Proc. Natl. Acad. Sci. U.S.A. 91*, 1515 −1518.

Borchert, T. V., Zeelen, J. P., Schliebs, W., Callens, M., Minke, W., Jaenicke, R., & Wierenga, R. K. (1995) *FEBS Lett. 367*, 315−318.

Breiter, D. R., Resnik, E., & Banaszak, L. J. (1994) *Protein Sci. 3*, 2023−2032.

Bullock, W. O., Fernandez, J. M., & Short, J. M. (1987) *BioTechniques 5*, 376−378.

Casal, J. I., Ahern, T. J., Davenport, R. C., Petsko, G. A., & Klibanov, A. M. (1987) *Biochemistry 26*, 1258−1264.

Chothia, C. (1991) *Curr. Opin. Struct. Biol. 1*, 53−59.

Dickason, R. R., & Huston, D. P. (1996) *Nature 379*, 652−655.
Erhardt, J., & Dirr, H. (1995) *Eur. J. Biochem. 230*, 614−620.
Fell, D. A., & Branford White, J. (1975) *Biochem. Biophys. Res. Commun. 67*, 1013−1018.
Gawehn, K., & Bergmeyer, H. U. (1974) in *Methoden der enzymatischen Analyse* (Bergmeyer, H. U., Ed.) pp 1542−1544, Verlag Chemie GmbH, Weinheim, Germany.
Goodsell, D. S., & Olson, A. J. (1993) *Trends Biochem. Sci. 18*, 65−68.
Higuchi, R. (1990) in *PCR protocols: a guide to methods and applications* (Innis, M. A., Gelford, D. H., Sinsky, J. J., & White, T. J., Eds.) pp 177−183, Academic Press, San Diego, CA.
Jaenicke, R. (1987) *Prog. Biophys. Mol. Biol. 49*, 117−237.
Jaenicke, R. (1996) *Curr. Top. Cell. Regul. 34*, 209−314.
Janin, J. (1995) *Biochemie 77*, 497−505.
Janin, J., & Chothia, C. (1990) *J. Biol. Chem. 265*, 16027−16030.
Janin, J., Miller, S., & Chothia, C. (1988) *J. Mol. Biol. 204*, 155−164.
Jones, S., & Thornton, J. M. (1995) *Prog. Biophys. Mol. Biol. 63*, 31−63.
Jones, T. A., Zou, J.-Y., Cowan, S. W., & Kjeldgaard, M. (1991) *Acta Crystallogr. 47A*, 110−119.
Knowles, J. R. (1991) *Nature 350*, 121−124.
Lambeir, A.-M., Opperdoes, F. R., & Wierenga, R. K. (1987) *Eur. J. Biochem. 168*, 69−74.
Mainfroid, V., Trepstra, P., Beauregard, M., Frere, J.-M., Mande, S. C., Hol, W. G. J., Martial, J. A., & Goraj, K. (1996) *J. Mol. Biol. 257*, 441−456.
Mossing, M. C., & Sauer, R. T. (1990) *Science 250*, 1712−1715.
Pekrun, A., Neubauer, B. A., Eher, S. W., Lakamek, M., Seidel, H., & Schröter, W. (1995) *Clin. Genet. 47*, 175−179.
Porvari, K. S., Herrala, A. M., Kurkela, R. M., Taavitsainen, P. A., Lindqvist, Y., Schneider, G., & Vikho, P. T. (1994) *J. Biol. Chem. 269*, 22642−22646.
Rietveld, A. W. M., & Ferreira, S. T. (1996) *Biochemistry 35*, 7743−7751.
Schliebs, W., Thanki, N., Eritja, R., & Wierenga, R. (1996) *Protein Sci. 5*, 229−239.
Steif, C., Weber, P., Hinz, H.-J., Flossdorf, J., Cesareni, G., & Kokkinidis, M. (1993) *Biochemistry 32*, 3867−3876.
Thanki, N., Zeelen, J. Ph., Mathieu, M., Jaenicke, R., Abagyan, R. A., Wierenga, R. K., & Schliebs, W. (1997) *Protein Eng. 10*, 159−167.
Vriend, G. (1990) *J. Mol. Graphics 8*, 52−56.
Ward, W. H. J., Hugh Jones, D., & Fersht, A. R. (1987) *Biochemistry 26*, 4131−4138.
Wierenga, R. K., Noble, M. E. M., & Davenport, R. C. (1992) *J. Mol. Biol. 224*, 1115−1126.
Yphantis, D. A. (1964) *Biochemistry 3*, 297−317.
Zabori, S., Rudolph, R., & Jaenicke, R. (1980) *Z. Naturforsch. 35C*, 999−1004.

BI963086A