An interface point-mutation variant of triosephosphate isomerase is compactly folded and monomeric at low protein concentrations

T.V. Borchert[a,**], J.Ph. Zeelen[a], W. Schliebs[a], M. Callens[b], W. Minke[b], R. Jaenicke[c], R.K. Wierenga[a,*]

[a]*EMBL, Postfach 102209, D-69012 Heidelberg, Germany*
[b]*Research Unit for Tropical Diseases, International Institute of Cellular and Molecular Pathology, Avenue Hippocrate 74, B1200, Brussels, Belgium.*
[c]*Institut für Biophysik und Physikalische Biochemie, Universität Regensburg, D-93040, Regensburg, Germany.*

Received 20 April 1995; revised version received 17 May 1995

Abstract Wild-type trypanosomal triosephosphate isomerase (wtTIM) is a very tight dimer. The interface residue His-47 of wtTIM has been mutated into an asparagine. Ultracentrifugation data show that this variant (H47N) only dimerises at protein concentrations above 3 mg/ml. H47N has been characterised at a protein concentration where it is predominantly a monomer. Circular dichroism measurements in the near-UV and far-UV show that this monomer is a compactly folded protein with secondary structure similar as in wtTIM. The thermal stability of the monomeric H47N is decreased compared to wtTIM: temperature gradient gel electrophoresis (TGGE) measurements give T_m-values of 41°C for wtTIM, whereas the T_m-value for the monomeric form of H47N is approximately 7°C lower.

Key words: Dimer; Triosephosphate isomerase; Interface; Ultracentrifugation; Monomer; Point mutation

1. Introduction

Oligomerisation is an important property of many polypeptides [1,2]. Some polypeptide chains which form oligomers display their function only as a monomer, for example insulin [3] and the catalytic subunit of cAMP dependent kinase [4]. In these examples the oligomerisation provides a storage form (insulin) or is used as a mechanism for regulation (the cAMP dependent kinase). Classical oligomers, such as most glycolytic enzymes are highly stable. They require the oligomeric state for their catalytic function; the monomeric forms, as well as their folding and assembly pathways, have yet to be characterised [5]. Our studies focus on the monomer/dimer properties of the dimeric glycolytic enzyme triosephosphate isomerase (TIM) from *Trypanosoma brucei brucei*. This enyzyme catalyzes the interconversion of dihydroxyacetone phosphate (DHAP) and D-glyceraldehyde-3-phosphate (GAP) [6].

The TIM dimer is an extremely tight dimer; for example, a constant specific activity has been measured in the concentration range from 1 to 2000 ng/ml [7]. The K_d of the wild-type protein has been estimated to be below 10^{-11} M [8]. It has been shown that the folding pathway of the TIM-dimer can be described by a consecutive first-order folding and second-order association reaction scheme assuming inactive monomers [7]. Under standard conditions the reaction is exceedingly fast so that the properties of the wild-type enyzme in its monomeric state have been inaccessible to a detailed analysis. The TIM subunit has the $(\beta/\alpha)_8$ fold with a central core of eight parallel β-strands (strand-1 to strand-8) covered on the outside by eight α-helices (helix-1 to helix-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. Loop-1 to loop-4 are additionally involved in the formation of the interface between the two subunits. Loop-3 is a long loop which protrudes 13 Å out of the bulk of the monomer and docks into a narrow pocket close to the active site of the other subunit. This loop is very important for the stability of the dimer since 80% of the intersubunit atom–atom contacts involve atoms of loop-3 [8]. The narrow pocket is shaped by loop-1 and loop-4. Because of these interdigitating loops at the dimer interface, it is not a priori obvious that the individual wild-type subunits will have a compactly folded structure by themselves. Previously, we have shown that the 15 residues of loop-3 of trypanosomal TIM (wtTIM) can be replaced by an eight residue loop, in such a way that a stable, monomeric protein (monoTIM) is obtained [8]. The crystal structure of monoTIM has been reported [9]. Here we report on the characterisation of another interface variant of wtTIM: H47N. In this variant an interface residue of loop-2, His-47, is mutated into an asparagine. In wtTIM the side chain of His-47 is involved in a water mediated hydrogen bond to Asp-85, which is located in helix-3 of the other subunit. This water molecule is fixed in a cavity completely buried at the dimer interface. His-47 is not close to any of the two active sites in the wtTIM dimer. The shortest distance of any atom of His-47 to the center of any of the two active sites is 16 Å. The center of the active site is defined as the position of a conserved water molecule, hydrogen bonded to the active site residues Asn-11 and His-95 [10]. Some properties of H47N have been described earlier [11].

H47N has been expressed in *E. coli*, purified, and enzymatically and biophysically characterized. Here it is shown by ultracentrifugal studies that H47N displays a monomer/dimer equilibrium being monomeric at low protein concentrations and dimeric at higher protein concentrations. The characterisa-

*Corresponding author. Fax: (49) (6221) 38-7306.

***Present address:* Novo-Nordisk A/S, Industrial Biotechnology, Novo Allé, DK-2880 Bagsvaerd, Denmark.

Abbreviations: Bis-Tris, bis[2-hydroxyethyl]imino-tris[hydroxymethyl]-methane; CD, circular dichroism; DHAP, dihydroxyacetone phosphate; DTT, dithiothreitol; EDTA, ethylenediamine tetraacetic acid; GAP, D-glyceraldehyde-3-phosphate; TGGE, temperature gradient gel electrophoresis; TIM, triosephosphate isomerase (EC 5.3.1.1); wtTIM, wild-type trypanosomal triosephosphate isomerase; 2PG, 2-phosphoglycollate.

SSDI 0014-5793(95)00586-2

tion of the monomeric form of H47N shows that this monomer is a compactly folded protein with secondary and tertiary structure similar to wtTIM. The catalytic activity as well as the thermal stability of this monomeric form are much decreased compared to the wild-type enzyme. These investigations show that suitable interface point mutations of TIM, despite their decreased stability, can be used to study the properties of such monomeric variants of TIM.

2. Materials and methods

The expression and purification of wtTIM and H47N have been described previously [11]. The expression was carried out in *E. coli* strain BL21(DE3) [12] transformed with a plasmid containing the TIM gene. The purification was carried out at 4°C with all buffers supplemented with 5 mM of DTT, and 1 mM of both EDTA, and NaN_3.

Triosephosphate isomerase activity was followed as the change of absorbance at 340 nm in a coupled enzyme assay as previously described [13]. One activity unit represents the conversion of one micromole substrate per minute at 25°C. For the K_m determinations the assay was initiated by adding TIM to the reaction mixture containing various amounts of substrate, i.e. 0.01–3.5 mM D-glyceraldehyde-3-phosphate or 0.2–0.6 mM dihydroxyacetone phosphate. The K_i of the substrate analogue 2-phosphoglycollate, 2PG, was obtained by analysing the effect of 2PG on the initial velocity of the conversion of D-glyceraldehyde-3-phosphate at 2PG concentrations between 0.0065 and 0.1 mM.

Sedimentation analysis was performed in a Beckman Spinco Model E analytical ultracentrifuge equipped with a UV scanning system. Double sector cells with saphire windows were used in AnF-Ti and AnG rotors. Sedimentation coefficients were determined from sedimentation velocity runs at 40,000–68,000 rpm, plotting log r versus time and correcting to 20°C and water viscosity. High-speed sedimentation equilibria according to Yphantis [14] were evaluated from ln c versus r^2 plots. The partial specific volume was calculated form the amino acid composition. Prior to the experiments, protein samples were equilibrated against a 50 mM sodiumphosphate buffer, pH 7.0, with 50 mM NaCl.

Circular dichroism (CD) measurements were carried out on a Jobin Yvon circular dichrometer model VI. All measurements were carried out in thermostated cuvettes at 20°C with a 1 cm path length for the near-UV region and an 0.02 cm pathlength for the far-UV region. The near-UV and far-UV spectra are normalised with respect to decimole of monomers and decimole of peptide bonds, respectively. Protein samples were equilibrated against a 50 mM sodiumphosphate buffer, pH. 7.0, supplemented with 20 mM NaCl.

3. Results

Based on enzyme activity measurements, we have earlier reported indirect evidence suggesting that H47N displays a monomer/dimer equilibrium being mostly monomeric at low protein concentrations and dimeric at high protein concentrations [11]. This hypothesis arose from the observation that the catalytic activity depends strongly on the protein concentration of H47N. At high protein concentrations the specific activity of H47N can be as high as 2300 units/mg, which is close to the specific activity of wtTIM, whereas at low protein concentrations it can be as low as 3 units/mg, suggesting that the H47N dimer is as active as wtTIM, but the H47N monomer is virtually inactive, compared to wild-type. The K_m-values for wild-type and H47N for both substrates are compared in Table 1. The K_m value for the isomerisation of GAP to DHAP by H47N has been determined at varying enzyme concentrations, but no effect of concentration is observed. In Table 1 are also compared the K_i-values for 2-PG. The close similarity of the K_m and K_i values of wtTIM and H47N, as well as the observation that the specific activity at high concentrations of H47N is close to wild-type, is in agreement with the assumption that the measured catalytic activity of H47N is due to the presence of active dimeric H47N.

Table 1
K_m and K_i values of wtTIM and H47N measured at 25°C

Enzyme	K_m (mM) GAP	K_m (mM) DHAP	K_i (mM) 2PG
wtTIM	0.25 ± 0.05[a]	1.2 ± 0.1[a]	0.027[a]
H47N	0.33 ± 0.04[b]	1.28 ± 0.07[b]	0.018

[a] As reported previously [13].
[b] As reported previously [11].

Sedimentation coefficients, $s_{20,w}$, from analytical ultracentrifugation experiments carried out with H47N at protein concentrations between 0.1 and 18 mg/ml are shown in Fig. 1. At protein concentrations below 1 mg/ml, a sedimentation coefficient $s_{20,w}$~2.5S was obtained which correlates well with standard globular proteins with a molecular mass of approximately 25 ± 5 kDa [14]. At elevated concentrations a plateau value of $s_{20,w}$ = 3.50 ± 0.04S is observed, independent of temperature (2–22°C). Taken together with the diffusion coefficient $D_{20,w} = 5.7 \pm 0.5 \times 10^{-7}$ $cm^2 \cdot s^{-1}$, obtained from synthetic boundary experiments at 2°C and 20°C, and the calculated partial specific volume (0.735 $cm^3 \cdot g^{-1}$), the Svedberg equation yields a molecular mass $M_{s,D}$ = 54.0 ± 4.6 kDa. This result is in good agreement with a dimeric quaternary structure of H47N at high protein concentration; at the same time, it is close to the value 55.6 ± 3.6 kDa observed for wtTIM. High speed equilibrium experiments at concentrations ranging from approximately 0.02–2 mg/ml yield a linear relationship between ln c (measured as absorbance at 280 nm and 235 nm) and r^2. From the slope a weight-average molecular mass of 28.7 kDa is calculated, in good agreement with a monomeric species.

As indicated by the foregoing results, H47N is monomeric at concentrations below 1 mg/ml. Thus one has to assume that during some stages of the purification most of the protein will be in the monomeric form, indicating that the H47N monomer must be a stable folded protein. To investigate this question further, the far-UV circular dichroism spectra were recorded for H47N and wtTIM at protein concentrations below 1 mg/ml, with the result that H47N is found to have a far-UV CD-spectrum that is closely similar to the one observed for wtTIM (Fig. 2A). From this finding we may conclude that most of the secondary structure elements found in wtTIM are conserved in

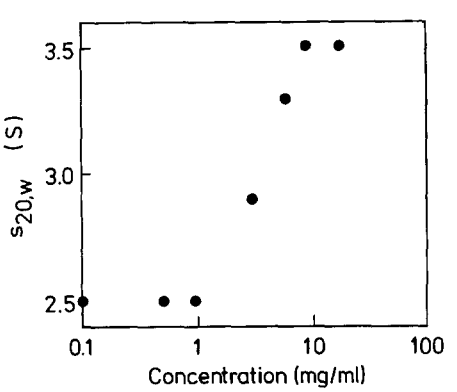

Fig. 1. Concentration dependence of $s_{20,w}$ of H47N from sedimentation velocity experiments at 40,000 and 68,000 rpm.

the mutant protein at a concentration where H47N is in its monomeric state.

The well-defined signal of the near-UV CD spectrum of monomeric H47N (Fig. 2B) shows that monomeric H47N has a compact tertiary structure. However, the comparison of the near-UV spectra of monomeric H47N and wtTIM clearly indicates that some aromatic residues must be in a different environment in the monomeric protein. The crystal structure of wtTIM shows that this could concern in particular Trp-12 in loop-1 and Tyr-101 and Tyr-102 in loop-4.

The thermal stability of wtTIM and H47N was compared by making use of the method of temperature gradient gel electrophoresis (TGGE) (Fig. 3A) as described previously for mono-TIM [8]. Since the protein concentration of the sample is approximately 1 mg/ml, the majority of the variant protein is dissociated into monomer. The monomeric form of H47N has a reduced thermal stability compared to the wild-type protein, in agreement with results obtained, for example for lactate dehydrogenase where a gradual decrease in stability was found in going from the natural tetramer to the 'proteolytic dimer' and finally to the monomer [16]. The TGGE experiment was repeated in the presence of 1 mM 2PG (Fig. 3B). The protein was equilibrated against the running buffer supplemented with 1 mM 2PG prior to the run. Under these conditions the T_m-values are increased by approximately 8°C, showing that the H47N monomer is stabilised by the substrate analogue, and indicating that the overall architecture of the triosephosphate isomerase active site is conserved in the monomeric state of H47N.

4. Discussion

In this study, the biophysical properties of an interface point-mutation variant of trypansomal TIM have been investigated.

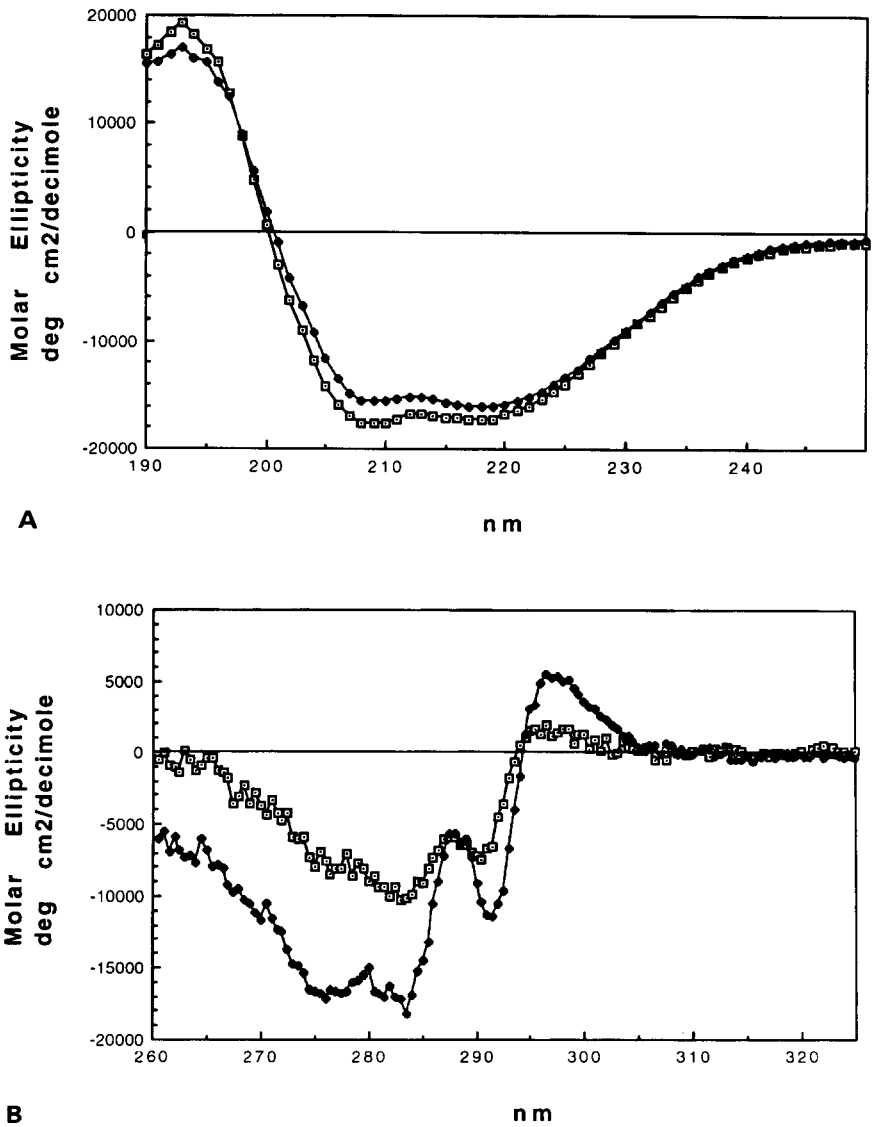

Fig. 2. Dichroic absorbtion spectra of wtTIM and H47N. (A) Far-UV CD spectra of wtTIM (◆) and H47N (□). The samples were diluted to an A_{280} = 0.40, which corresponds to approximately 0.3 mg/ml to obtain a good comparison of the spectra. (B) Near-UV CD spectra of wtTIM (◆) and H47N (□). The samples were diluted to an A_{280} = 0.80 (0.6 mg/ml) for optimal comparison.

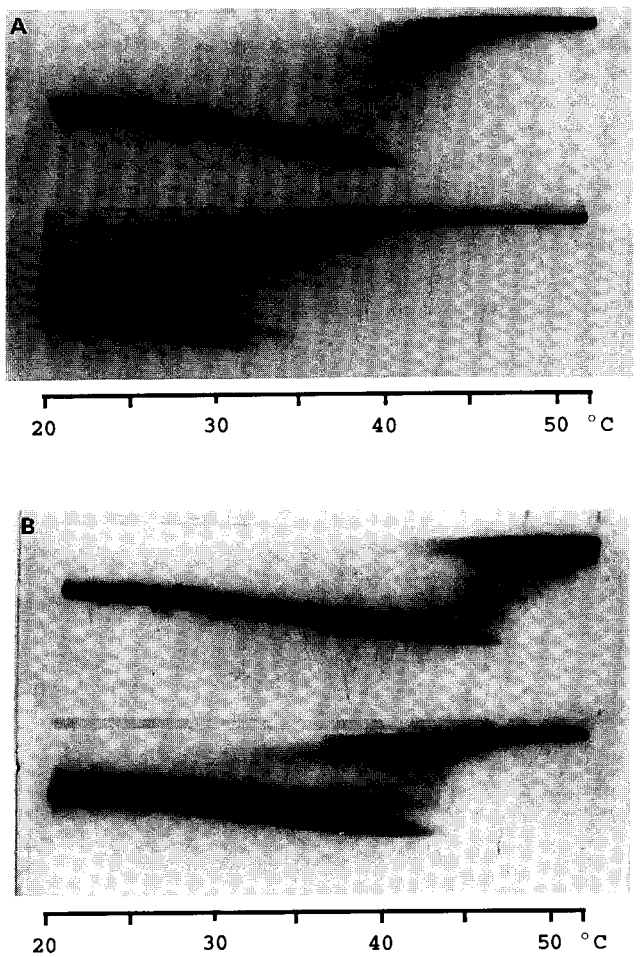

Fig. 3. Temperature gradient gel electrophoresis (TGGE, [8]) of wtTIM and H47N. The protein samples (approximately 1 mg/ml) were equilibrated against the running buffer (100 mM Bis-Tris/acetate, pH 7.0) and loaded across the gel: wtTIM (top) and H47N (below). The temperature gradient, as indicated below the gel, is formed perpendicular to the electric field. At high temperatures the proteins are denatured and aggregated and at low temperatures the protein migrates as the folded dimer (wtTIM) or monomer (H47N). The transition temperature (T_m) is a measure of the thermal stability. (A) In the absence of 2PG. The T_m-values are: wtTIM 41°C; and H47N 34°C. (B) In the presence of 1 mM 2PG. The T_m-values are: wtTIM 47°C; and H47N 43°C.

The H47N mutation disrupts an extensive buried hydrogen bonding network across the dimer interface, as has been discussed previously [11].

H47N is a monomer below 1 mg/ml and a dimer above 10 mg/ml (Fig. 1). Therefore the concentration at which 50% of the dimer is dissociated into monomer must be between 1 and 10 mg/ml. It is difficult to determine this point accurately from the available data. Taking the midpoint value of Fig. 1, which is $s_{20,w}$ = 3S at 3.6 mg/ml H47N, as a reference point at which 50% of the protein mixture is dimer, then the approximate value for K_d of the H47N-dimer is of the order of 5×10^{-5} M. Compared to wtTIM, with an estimated K_d below 10^{-11} M, at least a $5 \times 10^{+6}$-fold destabilization of the dimer is the result of this relatively modest alteration in the dimer interface.

The CD data (Fig. 2) and the TGGE-experiments (Fig. 3) strongly support the conclusion that the monomeric form of H47N is a stable, compactly folded protein with a partially intact active site architecture. Evidence for the occurrence of folded monomeric forms of yeast TIM has also been presented [17]; in this case an interface asparagine was changed into an aspartate. The observed binding of 2PG to monomeric H47N is not necessarily in contrast to its reduced catalytic activity; it is known that even a small relocation of catalytic residues in the TIM active site can cause a dramatic decrease in catalytic activity. For example the mutation E165D in yeast TIM (equivalent to E167D in wtTIM) reduces k_{cat} for the substrate GAP 2000-fold, whereas the K_i for the inhibitor phosphoglycolohydroxamate is decreased by a factor 3 [18], but only minor structural differences could be detected [19].

In wtTIM, extensive interactions are observed between the two subunits resulting in very tight dimers with a very low K_d. Since oligomerisation commonly leads to increased stability [16], it is not surprising that the monomeric form of H47N exhibits lower thermal stability than wtTIM. It is interesting to note that the thermal stability of this monomeric form (T_m = 34°C) is also reduced when compared with monoTIM, which has a T_m value of 38°C, as measured also with the TGGE technique [8]. Apparently the solvent exposed loop-3, which is present in monomeric H47N but not in monoTIM, destabilises the monomeric form of TIM.

Acknowledgements: We thank Prof. Dr. F.R. Opperdoes for his encouragement. This work was supported by an EC-BRIDGE grant (BIOT-CT90-0182).

References

[1] Goodsell, D.S. and Olson, A.J. (1993) Trends Biochem. Sci. 18, 65–68.
[2] Janin, J. (1991) Curr. Opin. Struct. Biol. 1, 42–44.
[3] Brange, J., Dodson, G.G. and Xiao, B. (1991) Curr. Opin. Struct. Biol. 1, 934–940.
[4] Taylor, S.S., Knighton, D.R., Zheng, J., Sowadski, J.M., Gibbs, C.S. and Zoller, M.J. (1993) Trends Biochem. Sci. 18, 84–89.
[5] Jaenicke, R. (1987) Prog. Biophys. Mol. Biol. 49, 117–237.
[6] Knowles, J.R. (1991) Nature 350, 121–124.
[7] Zabori, S., Rudolph, R. and Jaenicke, R. (1980) Z. Naturforsch. 35C, 999–1004.
[8] Borchert, T.V., Abagyan, R., Jaenicke, R. and Wierenga, R.K. (1994) Proc. Natl. Acad. Sci. USA 91, 1515–1518.
[9] Borchert, T.V., Abagyan, R., Radha Kishan, K.V., Zeelen, J.P. and Wierenga, R.K. (1993) Structure 1, 205–213.
[10] Wierenga, R.K., Borchert, T.V. and Noble, M.E.M. (1992) FEBS Lett. 307, 34–49.
[11] Borchert, T.V., Pratt, K., Zeelen, J.P., Callens, M., Noble, M.E.M., Opperdoes, F.R., Michels, P.A.M. and Wierenga, R.K. (1993) Eur. J. Biochem. 211, 703–710.
[12] Studier, F.W. and Moffatt, B.A. (1986) J. Mol. Biol. 189, 113–130.
[13] Lambeir, A.-M., Opperdoes, F.R. and Wierenga, R.K. (1987) Eur. J. Biochem. 168, 69–74.
[14] Yphantis, D.A. (1964) Biochemistry 3, 297–317
[15] Sober, H.A. (1970) in: Handbook of Biochemistry, Selected Data for Molecular Biology C10–12, The Chemical Rubber Co., Cleveland, OH.
[16] Jaenicke, R. (1991) Eur. J. Biochem. 202, 715–728.
[17] Casal, J.I., Ahern, T.J., Davenport, R.C., Petsko, G.A. and Klibanov, A.M. (1987) Biochemistry 26, 1258–1264.
[18] Blacklow, S.C. and Knowles, J.R. (1990) Biochemistry 29, 4099–4108.
[19] Joseph-McCarthy, D., Rost, L.E., Komives, E.A. and Petsko, G.A. (1994) Biochemistry 33, 2824–2829.