Subunit Interface of Triosephosphate Isomerase: Site-Directed Mutagenesis and Characterization of the Altered Enzyme†

Jose I. Casal,‡,§ Tim J. Ahern,§,∥ Robert C. Davenport,⊥ Gregory A. Petsko,*,# and Alexander M. Klibanov*,§

Departments of Applied Biological Sciences, Chemistry, and Biology, Massachusetts Institute of Technology, Cambridge, Massachusetts 02139

Received June 27, 1986; Revised Manuscript Received October 28, 1986

ABSTRACT: We have replaced asparagine residues at the subunit interface of yeast triosephosphate isomerase (TIM) using site-directed mutagenesis in order to elucidate the effects of substitutions on the catalytic activity and conformational stability of the enzyme. The mutant proteins were expressed in a strain of *Escherichia coli* lacking the bacterial isomerase and purified by ion-exchange and immunoadsorption chromatography. Single replacements of Asn-78 by either Thr or Ile residues had little effect on the enzyme's catalytic efficiency, while the single replacement Asn-78 → Asp-78 and the double replacement Asn-14/Asn-78 → Thr-14/Ile-78 appreciably lowered k_{cat} for the substrate D-glyceraldehyde 3-phosphate. The isoelectric point of the mutant Asn-78 → Asp-78 was equivalent to that of wild-type yeast TIM that had undergone a single, heat-induced deamidation, and this mutant enzyme was less resistant than wild-type TIM to denaturation and inactivation caused by elevated temperature, denaturants, tetrabutylammonium bromide, alkaline pH, and proteases.

Site-directed mutagenesis is an effective tool for probing the role specific amino acid residues play in the biological activity and conformational stability of proteins (Fersht et al., 1984; Smith, 1985). Using this technique, researchers have corrected deleterious mutations (Temple et al., 1982), modified enzyme–substrate affinity (Wilkinson et al., 1984), changed the pH dependence of enzymatic catalysis (Thomas et al., 1985), and stabilized enzymes against chemical oxidation (Estell et al., 1985) and thermoinactivation (Perry & Wetzel, 1984, 1986; Ahern et al., 1987). Future applications certainly will include modification of temperature optima, resistance to proteolysis, cofactor requirements, and allosteric regulation (Ulmer, 1983).

The success of such investigations depends upon the choice of replacement residues that strike the appropriate balance between conservation of the conformational integrity of the enzyme and promotion of the desired effect. Substitution of single amino acid residues at the subunit interface of oligomeric proteins can displace the equilibrium toward the monomeric forms (Perutz, 1978; Jones et al., 1985); therefore, they are excellent model systems for the study by classical methods of destabilizing mutations. In the present work, we have replaced amino acid residues at the subunit interface of the dimeric enzyme triosephosphate isomerase (EC 5.3.1.1) of the yeast *Saccharomyces cerevisiae* in order to elucidate how the character of a substituent affects conformational stability.

Yeast triosephosphate isomerase (53 000-dalton molecular mass) contains no cofactors, prosthetic groups, or metal atoms. It catalyzes the rapid interconversion of the unhydrated forms of D-glyceraldehyde 3-phosphate and dihydroxyacetone phosphate. The enzyme from yeast is moderately stable to temperature, pH, and denaturants (Waley, 1973). The three-dimensional structures of the enzyme from chicken muscle (Banner et al., 1975) and from yeast (Alber et al., 1981) have been determined by X-ray crystallography. The structure of the yeast enzyme has been refined at 1.9-Å resolution in the presence and absence of a transition-state analogue inhibitor (Davenport, 1986). The yeast enzyme has been the subject of a thorough investigation, combining X-ray crystallography and site-directed mutagenesis, of the relationship between the three-dimensional structure and enzymatic activity (Petsko et al., 1984). It is also an excellent system for detailed study of the importance of oligomeric interactions in protein stability and activity (Figure 1). The subunit interface is close to both active sites of the dimer and consists of a largely hydrophobic interior generated by a pair of interdigitating loops that proceed from each subunit to the back wall of the active site of the other subunit and then return (Alber et al., 1981). Residues in and around this loop are the targets for the studies reported herein.

Experimental Procedures

Materials

Phage M13mp18 and plasmid pUC18 were generously supplied by Dr. J. Messing of the Waksman Institute of Microbiology, Rutgers University (Norrander et al., 1983). The *Escherichia coli* strain JM101 (Δ*lac*, *pro*, *Sup*E, *thi*1, F′, *pro*AB$^+$, *lac* i$^+$, *lac* z m15 *tra* Δ36) was a gift of Dr. Regina Reilly, and the strain DF 502 [Δ(*rha*, *pfk*A, *tpi*) *pfk*B1, *his*$^-$, *pyr*D$^-$, *edd*$^-$, F$^-$, *str*^{r}] was a construct of Dr. Dan Fraenkel (Alber & Kawasaki, 1982).

Minimal medium is M9 medium (Miller, 1972) supplemented with 0.001% vitamin B_1 and 1% glycerol as the sole carbon source. Rich medium is LB medium (Maniatis et al., 1982) supplemented with 50 μg/mL ampicillin, 1% glycerol, and 1% lactose as the inducer.

Methods

Construction of Expression Vector. The system used to express the mutant proteins was obtained by selection of

† This work was supported by grants from the National Science Foundation (DMB-8520721) and Sun Oil Co.

‡ Fundacion Empresa Publica Postdoctoral Fellow. Present address: Ingenasa, c/ Hermanos Garcia Noblejas 42, Madrid, Spain.

§ Department of Applied Biological Sciences.

∥ Present address: Genetics Institute, Cambridge, MA 02140.

⊥ Department of Biology. Supported by NIH Training Grant GM07287.

Department of Chemistry.

0006-2960/87/0426-1258$01.50/0

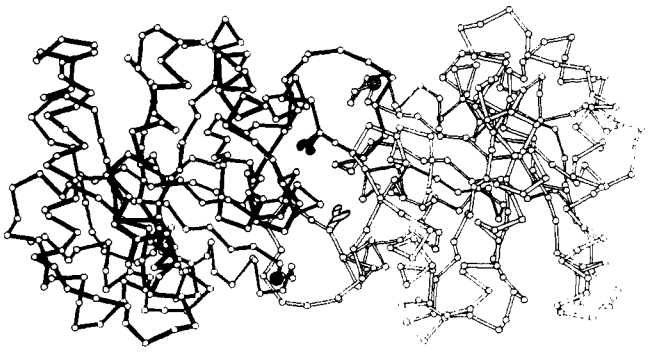

FIGURE 1: Drawing of the polypeptide chains of the dimeric enzyme yeast triosephosphate isomerase. The monomers are distinguished by filled or open interatomic connections. The α-carbon of Asn residue 14 and the side chain of Asn residue 78, indicated by circles and outlines, respectively, are distinguished as filled or striped. The interdigitating loops proceeding from each subunit are visible at the bottom and top of the intersubunit interface in the center.

fortuitous deletions (Alber & Kawasaki, 1982; Holland & Holland, 1980). A 3-kilobase *Pst*I–*Hin*dIII fragment of pTPIC10 (Alber & Kawasaki, 1982) containing the yTPI[1] gene was inserted into the homologously cleaved pUC18 (Norrander et al., 1983) using standard techniques (Maniatis et al., 1982). The resulting plasmid contained a unique *Pst*I site between the 3′ end of the *lac* promoter and the 5′ end of the yTPI coding region. The plasmid was purified from Rec$^+$ JM101 cells, 5 μg was digested with *Pst*I, and the resulting mixture was used to transform the *tpi*$^-$ *E. coli* strain DF502. *Amp*^{r} colonies, presumably containing plasmids in which the *Pst*I site had been deleted prior to digestion, were selected on rich medium minus lactose.

Comparison with DF 502 controls showed that ~30% of the *amp*^{r} transformants expressed the yTPI gene at levels sufficient to compensate the native deficiency. Colonies selected for assay of TIM activity were grown overnight in 1 mL of rich medium and pelleted by 12000*g* centrifugation for 1 min. The cells were resuspended in 0.1 mL of 50 mM triethanolamine hydrochloride buffer, pH 7.5, containing 1 mM EDTA and 1 mM β-mercaptoethanol and were made permeable by addition of 5 μL of toluene, followed by incubation at room temperature with agitation for 30 min. Subsequent enzymatic assay indicated clones contained levels of TPI expression as much as an order of magnitude higher than the control strain JM101. In addition, SDS–PAGE analyses (Laemmli, 1970) of these clones revealed a prominent band at the position of yTIM.

Restriction analysis of the plasmids expressing the highest levels of yTIM revealed a deletion between the 5′ end of the yTPI coding region and the 3′ end of the *lac* promoter of pUC18, resulting in the loss of a significant portion of the 5′ end of the yTPI promoter, most of the *lac* mRNA coding region, the *lacZ* Shine–Dalgarno sequence, and a portion of the *lac* operator (Figure 2a). One such plasmid was selected for sequence analysis and mutagenesis and is hereafter referred to as pUCyTPI.

Transfer of the yTPI Gene from Expression Vector pUCyTPI to M13mp18 for Mutagenesis. By means of established techniques (Maniatis et al., 1982), the region containing the LacPO and yTPI coding sequences was transferred from pUCyTPI to M13mp18; in short, pUCyTPI was cut with *Hae*II, the ends were blunted with Klenow fragment, and the resulting fragments were ligated into *Sma*I-digested M13mp18 during an 18-h incubation of 15 °C in the presence of T4 DNA ligase and ATP. Recombinant phage containing the yTPI gene was selected, and single-stranded phage and RF DNA were purified from the selected recombinants for use in mutagenesis (Norrander et al., 1983; Zoller & Smith, 1984).

Construction of Mutant Genes. The genes for mutant forms of triosephosphate isomerase (TIM) of the yeast *Saccharomyces cerevisiae* were constructed by the double-primer method of site-directed mutagenesis (Zoller & Smith, 1984). The nonmutagenic sequencing primers and mutagenic primers coding for the mutations Asn-14 → Thr-14 (5′GGAACCG*A*TTAATTT3′, where the asterisk denotes a mismatched base), Asn-14 → Asp-14 (5′GGAACCGT*C*TAATTT3′), Asn-78 → Ile-78 (5′AACGGAG*A*TTTCACC3′), Asn-78 → Thr-78 (5′AACGGAG*G*TTTCACC3′), and Asn-78 → Asp-78 (5′AACGGAGT*C*TTCACC3′) were synthesized by means of solid-phase phosphotriester chemistry on a DNA synthesizer (Biosearch Model SAM 1) and purified by gel electrophoresis (20% polyacrylamide–7 M urea; Maxam & Gilbert, 1980; Lo et al., 1984). A 5′-phosphorylated mutagenic primer (20 pmol) and a nonphosphorylated nonmutagenic primer (20 pmol) were mixed with single-stranded M13mp18 DNA (2 pmol), containing the yTPI gene, at 55 °C for 5 min in 10 mM Tris-HCl buffer, pH 8.5, containing 10 mM $MgCl_2$. The mismatched heteroduplexes were extended for 8 h at 15 °C in a reaction mixture containing 0.4 mM each of dATP, dGTP, dCTP, and dTTP, 0.5 mM ATP, DNA polymerase I Klenow fragment (0.1 unit/μL), T4 DNA ligase (0.15 unit/μL), 20 mM Tris-HCl buffer, pH 7.5, 10 mM $MgCl_2$, and 10 mM dithiothreitol in a total volume of 20 μL. Serial dilutions were used to transform competent *E. coli* JM101 cells as described previously by Zoller and Smith (1984).

Transformed plaques were isolated and grown in 1-mL cultures of *E. coli* JM101 in minimal medium for 6 h at 37 °C; then the clarified phage was adsorbed to nitrocellulose paper and baked in vacuo at 75 °C for 1.5 h prior to hybridization with isotopically labeled mutagenic oligonucleotide. The DNA of positive plaques selected after three rescreenings (Zoller & Smith, 1984) was purified, sequenced by the dideoxy sequencing method (Sanger et al., 1977), and found to contain only the expected base pair substitutions. The double-stranded DNA of positive plaques was purified by alkaline treatment and extraction (Birnboim & Doly, 1979), and the gene encoding mutant TIM was excised by endonucleases *Ava*I and *Cla*I. The excised gene was then inserted into phosphatase-treated pUC18 previously digested with *Ava*I and *Acc*I. Competent cells of *E. coli* DF502 lacking the gene for wild-type bacterial TIM were transformed with the modified pUC plasmid containing mutant TIM. The resulting transformants were tested for growth on ampicillin plates and the presence of enzymatic activity. Presence of the mutant gene was verified by sequence analysis (Wallace et al., 1981) of the plasmid purified from batch fermentations.

Preparation of TIM Antibodies for Immunoadsorption. Three sequential intramuscular inoculations of two female

[1] Abbreviations: ATP, adenosine 5′-triphosphate; dATP, 3′-deoxyadenosine 5′-triphosphate; dCTP, 3′-deoxycytidine 5′-triphosphate; dGTP, 3′-deoxyguanosine 5′-triphosphate; dTTP, 3′-thymidine 5′-triphosphate; EDTA, ethylenediaminetetraacetic acid; HPLC, high-performance liquid chromatography; NADH, nicotinamide adenine dinucleotide, reduced; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline; PMSF, phenylmethanesulfonyl fluoride; RF, replicative form of phage M13mp18; SDS, sodium dodecyl sulfate; TEMED, *N,N,N′,N′*-tetramethylethylenediamine; TIM, triosephosphate isomerase; yTPI, gene coding for triosephosphate isomerase of the yeast *Saccharomyces cerevisiae*; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride.

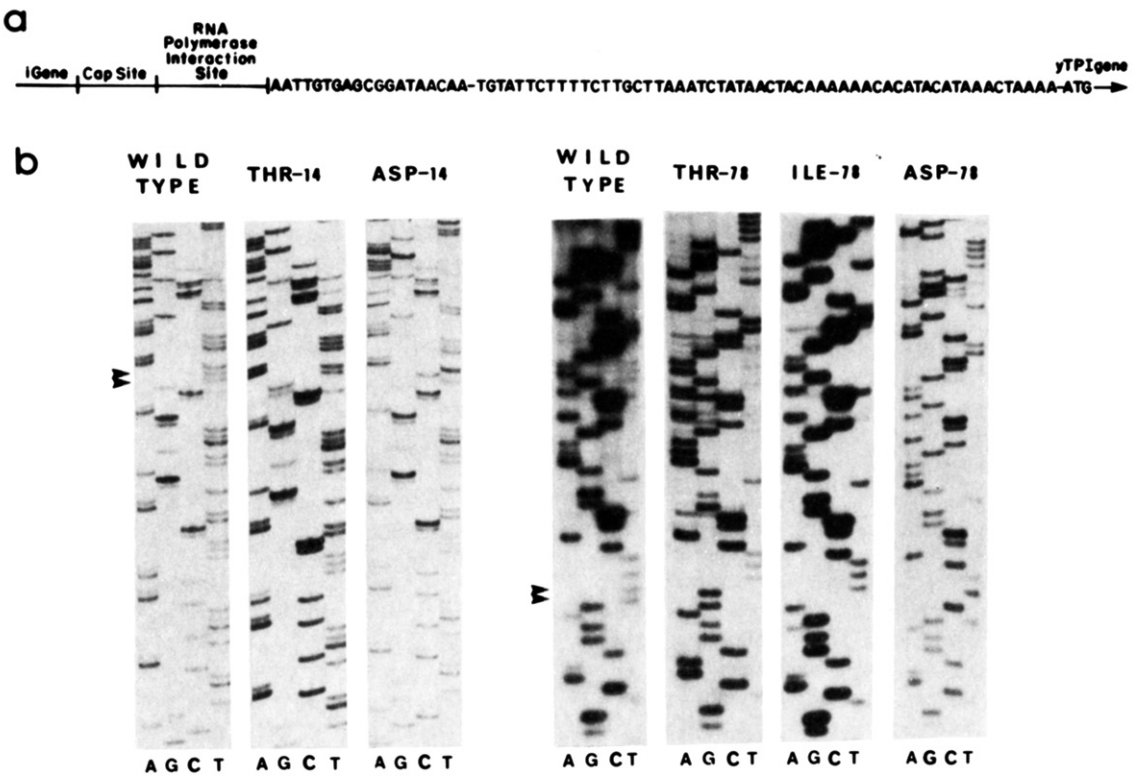

FIGURE 2: DNA sequences of the 5′ end (a) and the regions of mutation (b) of the yTPI gene expressed by pUC19 in *Escherichia coli*. (a) Sequence at the 5′ end of the yTPI gene after deletion of the *lac* mRNA coding region and flanking sequences. (b) Sequences of regions of the yTPI gene coding for the mutations at the Asn-14 and Asn-78 codons. The positions of base changes are indicated by arrows. See Methods for details.

NZW rabbits were performed: one every 3 weeks with 250 μg of purified yeast TIM (Sigma type 1) as an emulsion in (1) complete Freund's adjuvant, (2) incomplete Freund's adjuvant, and finally in (3) PBS. The animals were bled 10 days after the last immunization, and the antibodies specific for yeast TIM were detected by ELISA assay (Voller et al., 1979) and purified according to the method of Axen et al. (1967): A sample of rabbit serum (15 mL) containing TIM antibodies was passed through a column containing yeast TIM covalently bound to CNBr-activated Sepharose CL-4B. The column was subsequently washed with PBS, followed by 3 M KSCN to elute the antibodies. The eluate was dialyzed prior to attachment of the antibody to CNBr-activated Sepharose 4B, and the immobilized antibody was packed in columns for use in the purification of yeast TIM.

Production and Purification of TIM. All steps of purification were monitored by SDS–PAGE (15% acrylamide) using the buffer system of Laemmli (1970). Approximately 25–30 g (wet weight) of DF 502 *E. coli* cells was harvested from 9.9 L of culture grown to 6 OD_{600} units in shaking flasks in rich medium. Cells were lysed by treatment with Triton X-100 (0.1%) and lysozyme (0.5 mg/mL). The lysate was centrifuged at 30 000 rpm for 120 min in a Ti 45 rotor (Beckman) to remove cell debris. The 35–95% saturated ammonium sulfate fraction of the clarified lysate was dialyzed against 0.1 M phosphate buffer, pH 7.4, containing 0.15 M NaCl (PBS) (3 × 2 L). The insoluble material was subsequently removed by centrifugation, and the supernatant was applied to a DEAE-cellulose (Sigma) column and eluted with PBS. The pooled fractions containing TIM activity were concentrated before subjection to immunoadsorption chromatography on immobilized antibodies specific for yeast TIM. The protein was eluted with 3 M KSCN and dialyzed immediately against 0.1 M phosphate buffer, pH 6 (3 × 2 L). The final enzyme yields were typically 10–12 mg of TIM of greater than 95% purity, as determined by SDS–PAGE, isoelectrofocusing, and nondenaturing molecular sieve chromatography (Superose 12 HR 10/40, Pharmacia).

Activity Assay for Triosephosphate Isomerase. The enzyme-linked spectrophotometric assay based on the decrease of absorbance at 340 nm due to the oxidation of NADH (ϵ = 6220 M^{-1} cm^{-1}) was used to determine the activity of TIM (Hartman & Norton, 1975). In the presence of TIM, D-glyceraldehyde 3-phosphate is converted to dihydroxyacetone phosphate, whose reduction to α-glycerol 3-phosphate is catalyzed by α-glycerophosphate dehydrogenase with concomitant oxidation of NADH. Samples of TIM (10 μL) were mixed in a solution containing D-glyceraldehyde 3-phosphate (1 mM), 10 μg/mL α-glycerophosphate dehydrogenase (type 1, Sigma), EDTA (5 mM), and NADH (0.1 mg/mL) in 20 mM triethanolamine hydrochloride buffer, pH 7.9. One unit of enzymatic activity is the conversion of 1 μmol of D-glyceraldehyde 3-phosphate to dihydroxyacetone phosphate per minute.

Kinetic Studies. Kinetic measurements were carried out according to the method of Plaut and Knowles (1972) in a Model 552 spectrophotometer coupled to a Model 561 recorder (Perkin Elmer) at 30 °C maintained by a circulating constant-temperature bath. The cuvette contained 0.1 M buffer solution (cacodylate and triethanolamine hydrochloride for pH 6 and 7.9, respectively), 5 mM EDTA, 0.2 mM NADH, 0.017 mg/mL α-glycerophosphate dehydrogenase (Sigma, dialyzed to remove ammonium sulfate), 0.3–2.0 mM D-glyceraldehyde 3-phosphate, and 1–25 ng/mL TIM to initiate the reaction. The total volume was 1.5 mL. The kinetic parameters k_{cat} (calculated per dimer) and K_m were obtained from unweighted least-squares analysis of plots of v_0 vs. $v_0/[S_0]$ (the slope of which gives K_m) and $[S_0]$ vs. $[S_0]/v_0$ (the slope of which gives k_{cat}), where v_0 is the initial velocity and $[S_0]$ is the initial substrate concentration. The tabulated values for K_m were

calculated on the basis that only 4% of the substrate is reactive, since approximately 96% is hydrated under the conditions of the experiment (Reynolds et al., 1971).

PAGE. Enzyme preparations were applied to vertical slab gels [15% acrylamide:0.4% bis(acrylamide) ratio] and subjected to electrophoresis at 40 mA at constant temperature.

SDS–PAGE. Enzyme preparations were mixed with 80 mM Tris–H_3PO_4 loading buffer (pH 6.8) containing 1.3% SDS and 0.13% β-mercaptoethanol, 13% glycerol, and 0.01% bromphenol blue, heated at 100 °C for 1 min, and subjected to electrophoresis at 20 mA in vertical slab gels (Laemmli, 1970).

Isoelectrofocusing. Enzyme preparations containing 10% urea were applied to vertical slab gels (prefocused for 1 h at 200 V) containing Ampholines pH 5–7 (0.9% w/v, LKB), 3.5% acrylamide, 0.2% bis(acrylamide), 48% urea, 0.01% TEMED, and 0.002% ammonium persulfate. Protein in all gels was visualized by staining with Coomassie Blue (0.025%) in H_2O–methanol–acetic acid (50:40:10 by volume).

Molecular Sieve Chromatography. Purified enzyme (0.1 mg/mL) was applied to a Superose 12 HR 10/30 column and eluted at 23 °C and a flow rate of 0.5 mL/min by means of FPLC (Pharmacia). The elution buffers used were 0.1 M cacodylate (pH 6), triethanolamine (pH 7.9), or glycine (pH 9.5) containing 5 mM EDTA and urea ranging from 0 to 8 M concentration.

Proteolytic Digestion. Wild-type TIM, wild-type TIM carboxymethylated according to the procedure of Crestfield et al. (1963), and mutant Asn-78 → Asp-78 (500 μg of each) were separately treated with trypsin, chymotrypsin, and subtilisin at TIM:protease ratios of 100:1 (w/w). The incubation was typically performed in 0.1 M phosphate buffer at the pH indicated and 37 °C. Aliquots were withdrawn periodically, stored on ice after addition of the protease inhibitor PMSF (1 mM), and assayed for activity. Aliquots were then analyzed for proteolysis by SDS–PAGE [20% acrylamide–0.5% bis(acrylamide)] and by reverse-phase chromatography.

Reverse-Phase Chromatography of Tryptic Peptides. Digested samples of TIM were subjected to HPLC analysis by means of a 3.9 × 300 mm μBondapak C18 column (Waters Associates). Solvent A was 0.1% trifluoroacetic acid, and solvent B was trifluoroacetic acid–water–acetonitrile (0.1:9.9:90). A linear gradient from 0 to 70% solvent B within 90 min was applied, and the absorbance of the eluted peptides was monitored at 206 nm.

Results and Discussion

For the purpose of engineering a viable expression vector for TIM, the fragment of a previously constructed plasmid (pTPIC10) (Alber & Kawasaki, 1982) that contained the yTPI gene was inserted into a plasmid (pUC18) useful for the expression of foreign protein in *E. coli* cells. By selection of fortuitous deletions, we isolated a pUC18 plasmid that bore the gene for wild-type yeast TIM under the control of the *lac* operon (Figure 2a). *E. coli* containing the modified pUC plasmid expressed wild-type TIM in sufficient quantity for the following experiments.

By means of site-directed mutagenesis, we produced mutant TPI genes in which the wild-type codons (AAT) specifying Asn residues 14 and 78 were replaced for the production of mutants Asn-78 → Thr-78, Asn-78 → Ile-78, Asn-78 → Asp-78, and Asn-14/Asn-78 → Thr-14/Ile-78 and Asp-14/Asp-78 (Figure 2b).

The wild-type and mutant proteins were produced in batch fermentations. With the exception of cultures of the double mutant Asp-14/Asp-78, cell lysates typically yielded 10 000–40 000 units of TIM activity per liter of culture. More than 84% of the activity was subsequently recovered during purification of TIM to greater than 95% purity, as determined by SDS–PAGE, PAGE, isoelectrofocusing, and molecular sieve chromatography (Figure 3a–e). The mutants were composed of ~26 000-dalton monomers, as shown by SDS–PAGE (Figure 3c), which remained associated as dimers in the pH range 6.0–9.5 at 4–23 °C as determined by molecular sieve chromatography (data not shown) and by nondenaturing PAGE (Figure 3d). As shown in Figure 3e, the isoelectric points of the mutants Asn-78 → Thr-78, Asn-78 → Ile-78, and Asn-14/Asn-78 → Thr-14/Ile-78 are indistinguishable from that of native TIM (~5.3). Because of the presence of an extra anionic group, the isoelectric point of the mutant Asn-78 → Asp-78 is lower than that of the wild-type TIM (Figure 3e), and therefore, it travels in isoelectrofocusing gels similar to TIM subunits that have undergone a single Asn deamidation due to heating (Ahern et al., 1987).

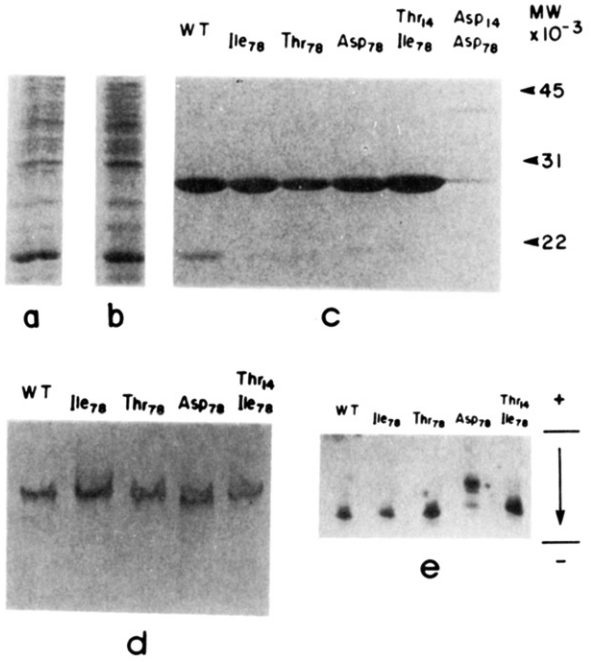

FIGURE 3: Purification and characterization of wild-type and mutant forms of TIM expressed in *E. coli*. SDS–PAGE of cell lysate (a), the active fraction after DEAE-cellulose chromatography (b), and TIM after immunoadsorption chromatography (c). Nondenaturing PAGE, pH 6 (d), and isoelectrofocusing (e) of wild-type and mutant forms of TIM.

In contrast, lysates of cultures of *E. coli* that contained pUC18 bearing the gene coding for the double mutant Asn-14/Asn-78 → Asp-14/Asp-78 (as verified by plasmid purification and sequencing) exhibited only a trace of TIM activity (less than 50 units/L), and soluble enzyme was barely detectable by the standard method of purification (Figure 3c).

The purified, concentrated forms of TIM (>5 mg/mL) were stable at 10 °C in 0.1 M phosphate buffer, pH 6, containing 8.4 mM EDTA: no irreversible dissociation, as determined by molecular sieve chromatography, or loss of enzymatic activity was observed during storage for several months.

The kinetic parameters of the wild-type and mutant forms of TIM are given in Table I. Under the conditions used, linear reciprocal plots are obtained that yield values for K_m and k_{cat}. Comparison of values for k_{cat}/K_m reveals that substitution of Asn-78 by either Ile or Thr has little effect on the catalytic efficiency of the enzyme. These unchanged residues approximate the geometry of the substituted Asn residue, and their

Table I: Kinetic Parameters of Mutant Triosephosphate Isomerase Activity for Glyceraldehyde 3-Phosphate

TIM[a]	pH	k_{cat} ($\times 10^{-4}$ s^{-1})	K_m (μM)	k_{cat}/K_m ($\times 10^{-8}$ $M^{-1} \cdot s^{-1}$)
wild type	6	1.95	46	4.2
Ile-78		1.72	42	4.1
Thr-78		1.78	38	4.7
Asp-78		1.29	38	3.4
Thr-14/Ile-78		1.15	49	2.3
wild type	7.9[b]	2.27	42	5.4
Ile-78		2.34	46	5.1
Thr-78		1.68	42	4.0
Thr-14/Ile-78		1.31	49	2.7

[a] An attempt to make a double mutant Asn-14/Asn-78 → Asp-14/Asp-78 yielded only a trace of enzymatic activity and no soluble enzyme upon purification, despite the presence of mutant plasmid in the cells. See text for discussion. [b] At pH 7.9, the Asn-78 → Asp-78 TIM was unstable in the enzyme concentration range suitable for kinetic analysis and hence was not investigated.

hydrophobic character evidently makes them good replacement residues for positions in the subunit interface of the enzyme.

The mutant Asn-78 → Asp-78 is unstable at low protein concentrations (<50 ng/mL) at pH 7.9; at pH 6, its k_{cat} is two-thirds of that of wild-type TIM. Substitution of an Asn by an Asp residue models the effects of deamidation of a single residue in a protein. Previous studies have shown that isolated monodeamidated forms of cytochrome *c* (Flatmark, 1967), lysozyme (Ahern & Klibanov, 1985), and ribonuclease A (Zale & Klibanov, 1986) exhibit biological/specific activities of approximately 60%, 55%, and 65%, respectively, of that of the wild-type forms.

In an effort to determine the effect of cumulative replacement at the interface on the catalytic efficiency, it was observed that k_{cat}/K_m decreased approximately by half relative to wild type at pH 6 and 7.9 when Asn-14 and Asn-78 were both replaced by Thr and Ile, respectively (Table I). However, unlike the Asn-78 → Asp-78 mutant, the doubly altered enzyme was not subject to dilution-induced inactivation at mildly alkaline pH. We therefore ascribe the instability of the Asn-78 → Asp-78 mutant to the unfavorable thermodynamics of dimerization due to the presence of a potentially charged residue at a normally buried interface.

The reduced stability of the Asn-78 → Asp-78 mutant was illustrated by its susceptibility to denaturation by urea. Denatured, dissociated forms of TIM elute from molecular sieve chromatographic columns earlier than the native dimer (Figure 4a). This transition was observed to occur at <2.5, 3, 5, and >8 M urea for Asn-78 → Asp-78, Asn-14/Asn-78 → Thr-14/Ile-78, Asn-78 → Ile-78, and wild-type forms of TIM, respectively. Dissociation of the Asn-78 → Asp-78 mutant enzyme to less denatured, or nondenatured, monomer, indicated by the appearance of a peak of protein eluting later than native dimer, appeared to occur at 1.5 M urea (Figure 4a). This transition was not observed in wild type or the other mutants. We hypothesize that the presence of a charged Asp residue at the normally buried interface shifts the thermodynamic equilibrium to the monomeric state at urea concentrations too low to induce a massive loss of ordered structure.

This view is also supported by the fact that the "activity vs. pH" profile of the Asn-78 → Asp-78 mutant is shifted 0.5 unit to the left in the alkaline range: whereas wild-type TIM and the other mutants exhibit half of their maximal activity at pH 9.5, the Asp mutant does so at pH 9.0.

To further establish the enhanced susceptibility of the Asn-78 → Asp-78 mutant to dissociation, the mutants were incubated in the presence of a lyotropic salt, tetrabutylammonium bromide. It was reasoned that the association of the bulky cation with the charged Asp-78 residue at the interface would result in the dissociation of the Asp mutant enzyme to stable monomer. In the presence of 120 mM tetrabutylammonium bromide, the Asp mutant retains only 5% residual activity, whereas the wild-type and other monosubstituted mutant forms exhibit 10-fold greater residual activity. In addition, the elution profile of native TIM is unaffected by the presence of the lyotropic salt, whereas the Asn-78 → Asp-78 mutant appears to elute as a mixture of dimer and monomer during molecular sieve chromatography (Figure 4a).

When the temperature at which nondenaturing gel electrophoresis is performed is increased from 4 to 50 °C, the mutant forms no longer run as native dimers (Figure 3d) but as unfolded forms having higher frictional coefficients (Figure 4b). Since elevated temperature, like urea, induces transitions

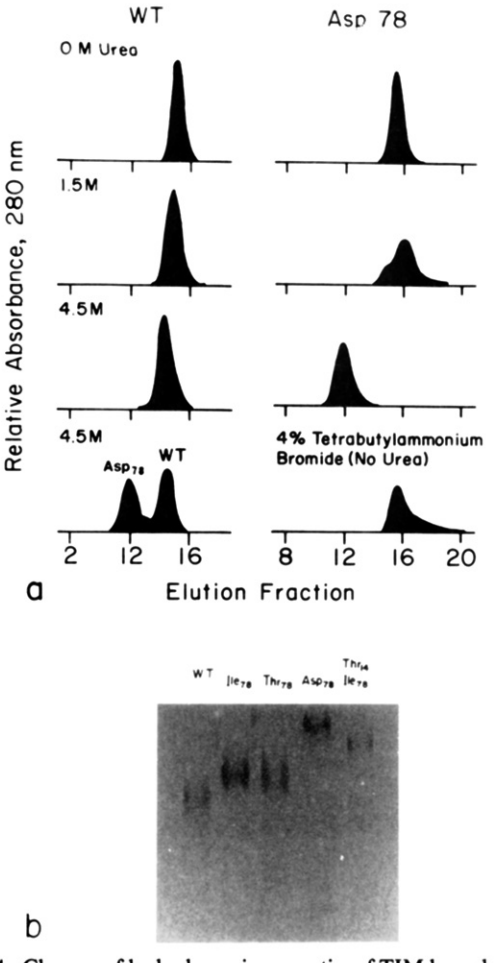

FIGURE 4: Changes of hydrodynamic properties of TIM brought about by site-directed mutagenesis. (a) Molecular sieve chromatography of native TIM and the Asn-78 → Asp-78 mutant in the presence of urea and 4% (120 mM) tetrabutylammonium bromide. The left column represents the elution of wild-type TIM from Superose 12 HR 10/30 FPLC columns equilibrated with the urea concentrations indicated. The bottom left elution profile also contained Asn-78 → Asp-78 mutant TIM. The right column represents the elution of Asn-78 → Asp-78 mutant TIM from columns equilibrated with the same urea concentrations as those in the left column, with the exception of the bottom right elution profile which contained 4% tetrabutylammonium bromide and no urea. The elution of TIM was monitored by means of UV absorbance at 280 nm. Folded monomeric forms, due to their smaller hydrodynamic volume, elute later than the wild-type dimer. In contrast, the unfolded monomer elutes earlier than the wild-type TIM, presumably because of its much greater hydrodynamic volume. (b) Native gel electrophoresis of wild-type and mutant TIM at 50 °C, pH 6. See Methods for details.

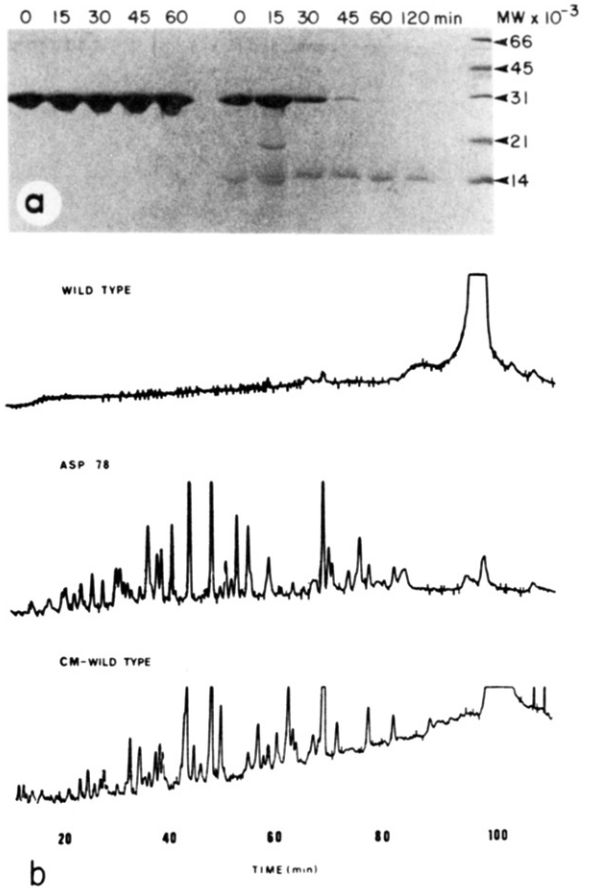

FIGURE 5: Proteolysis of TIM by trypsin. (a) SDS–PAGE of wild-type TIM (left), and mutant Asn-78 → Asp-78 (right) after incubation at pH 8 and 37 °C with trypsin for the time indicated. (b) HPLC profile of tryptic digests (37 °C, 20 h) of wild-type, carboxymethylated wild-type, and Asn-78 → Asp-78 mutant TIM.

resulting in disordered conformations, the relative mobilities reflect the decreasing order of transition temperatures of 60, 58, 57, 48 and 44 °C for wild-type TIM and the mutants Asn-78 → Thr-78, Asn-78 → Ile-78, Asn-14/Asn-78 → Thr-14/Ile-78, and Asn-78 → Asp-78, respectively (Ahern et al., 1987).

The introduction of an Asp residue at the subunit interface markedly increased the rate of proteolysis of TIM by trypsin (Figures 5 and 6). A decrease in the molecular weight of mutant TIM, as determined by SDS–PAGE, is observed in the course of incubation in the presence of trypsin at 37 °C; these conditions resulted in no detectable proteolysis of wild-type TIM (Figure 5a). The tryptic digest of the mutant Asn-78 → Asp-78, separated by means of reverse-phase HPLC, is shown in Figure 5b. Although folded wild-type TIM incubated in the presence of trypsin elutes as a single peak, once unfolded by carboxymethylation of its cysteinyl residues, wild-type TIM undergoes proteolysis as well (Figure 5b).

Proteolysis by trypsin, subtilisin, or chymotrypsin results in a loss of enzymatic activity of the Asn-78 → Asp-78 mutant that follows first-order kinetics; in contrast, wild-type TIM still retained more than 95% of its activity after a period of time that resulted in as little as 5% residual activity for the Asp mutant (Figure 6a). The Asn-78 → Asp-78 mutant also lost its activity during incubation in the presence of cell lysates of *E. coli* (Figure 6b). This finding supports the proposal that deamidation can serve as an in vivo biological timer of protein turnover (Robinson et al., 1970). We hypothesize that the low yields of TIM activity from fermentations of *E. coli* containing plasmid expressing the double mutant Asn-14/

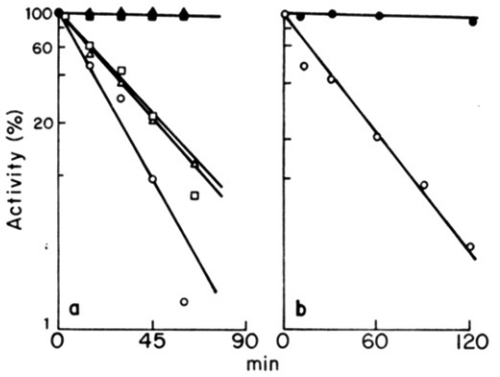

FIGURE 6: Activity of wild-type and mutant TIM in the presence of proteases (a) and cell lysate of *E. coli* (b). Wild-type TIM (closed symbols) and Asn-78 → Asp-78 mutant TIM (open symbols) were incubated at pH 8 and 37 °C with trypsin (○), subtilisin (△), and α-chymotrypsin (□). (b) The same forms of TIM were incubated in the cell lysate of *E. coli* at 37 °C. See Methods for details.

Asn-78 → Asp-14/Asp-78 are due to the low in vivo conformational stability of the mutant TIM, which results in its proteolytic digestion within the cell.

The foregoing results support the view that replacements conserving the geometry and charge of a residue (in this case, Asn) can be made without affecting significantly the catalytic efficiency of an enzyme. However, even moderate changes such as Asn → Ile or Thr can compromise the stability of the enzyme with respect to certain denaturing conditions, e.g., low concentrations of urea. More radical replacements such as Asn → Asp, which in this case resulted in the presence of a charged group at a normally buried, hydrophobic surface, significantly lowered the stability of the enzyme with respect to a host of denaturing conditions. However, it is important to note that such a replacement need not result in the complete inactivation of an enzyme or even disrupt its quaternary structure under normal conditions. This observation illustrates a tolerance to perturbation that speaks well for the future of practical applications of site-directed mutagenesis.

REFERENCES

Ahern, T. J., & Klibanov, A. M. (1985) *Science* (*Washington, D.C.*) *228*, 1280–1284.

Ahern, T. J., Casal, J. I., Petsko, G. A., & Klibanov, A. M. (1987) *Proc. Natl. Acad. Sci. U.S.A.* (in press).

Alber, T., & Kawasaki, G. (1982) *J. Mol. Genet. 1*, 419–434.

Alber, T., Banner, D. W., Bloomer, A. C., Petsko, G. A., Phillips, D. C., Rivers, P. S., & Wilson, I. A. (1981) *Philos. Trans. R. Soc. London, B 293*, 159–171.

Axen, R., Porath, J., & Ernback, S. (1967) *Nature* (*London*) *214*, 1302–1304.

Banner, D. W., Bloomer, A. C., Petsko, G. A., Phillips, D. C., Pogson, C. I., Wilson, I. A., Corran, P. H., Furth, A. J., Milman, J. D., Offord, R. E., Priddle, J. D., & Waley, S. G. (1975) *Nature* (*London*) *255*, 609–614.

Birnboim, H. C., & Doly, J. (1979) *Nucleic Acids Res. 7*, 1513–1523.

Crestfield, A. M., Moore, S., & Stein, W. H. (1963) *J. Biol. Chem. 238*, 622–627.

Davenport, R. C. (1986) Ph.D. Dissertation, Massachusetts Institute of Technology, Cambridge, MA.

Estell, D. A., Graycar, T. P., & Wells, J. A. (1985) *J. Biol. Chem. 260*, 6518–6521.

Fersht, A. R., Shi, J., Wilkinson, A. J., Blow, D. M., Carter, P., Waye, M. M. Y., & Winter, G. P. (1984) *Angew. Chem. 23*, 467–473.

Flatmark, T. (1967) *J. Biol. Chem. 242*, 2454–2459.

Hartman, F. C., & Norton, I. L. (1975) *Methods Enzymol. 41*, 447–453.
Holland, J. P., & Holland, M. J. (1980) *J. Biol. Chem. 255*, 2596–2605.
Jones, D. H., McMillan, A. J., & Fersht, A. R. (1985) *Biochemistry 24*, 5852–5857.
Laemmli, U. K. (1970) *Nature (London) 227*, 680–685.
Lo, K. M., Jones, S. S., Hacket, N. R., & Khorana, H. G. (1984) *Proc. Natl. Acad. Sci. U.S.A. 81*, 2285–2289.
Lu, H. S., Yuan, P. M., & Gracy, R. (1984) *J. Biol. Chem. 259*, 11958–11968.
Maniatis, T., Fritsch, E. F., & Sambrook, J. (1982) *Molecular Cloning, A Laboratory Manual*, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
Maxam, A. M., & Gilbert, W. (1980) *Methods Enzymol. 65*, 499–560.
Miller, J. H. (1972) *Experiments in Molecular Genetics*, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
Norrander, J., Kempe, T., & Messing, J. (1983) *Gene 26*, 101–106.
Perry, L. J., & Wetzel, R. (1984) *Science (Washington, D.C.) 226*, 555–557.
Perry, L. J., & Wetzel, R. (1986) *Biochemistry 25*, 733–739.
Perutz, M. F. (1978) *Science (Washington, D.C.) 201*, 1187–1191.
Petsko, G. A., Davenport, R. C., Fraenkel, D., & Rajbhandary, U. L. (1984) *Biochem. Soc. Trans. 12*, 229–232.
Plaut, B., & Knowles, J. R. (1972) *Biochem. J. 129*, 311–320.
Reynolds, S. J., Yates, D. W., & Pogson, C. J. (1971) *Biochem. J. 122*, 285–297.
Robinson, A. B., McKerrow, J. H., & Cary, P. (1970) *Proc. Natl. Acad. Sci. U.S.A. 66*, 753–757.
Sanger, F., Nicklen, S., & Coulson, A. R. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74*, 5463–5469.
Smith, M. (1985) *Annu. Rev. Genet. 19*, 423–463.
Temple, G. F., Dozy, A. M., Roy, K. L., & Kan, Y. W. (1982) *Nature (London) 296*, 537–539.
Thomas, P. G., Russell, A. J., & Fersht, A. R. (1985) *Nature (London) 318*, 375–376.
Ulmer, K. M. (1983) *Science (Washington, D.C.) 219*, 666–671.
Voller, D., Bidwell, D. E., & Bartlett, D. (1979) *The Enzyme-linked Immunosorbent Assay (ELISA). A Guide with Microplate Applications*, Flowline Press, Guernsey, U.K.
Waley, S. G. (1973) *Biochem. J. 135*, 165–172.
Wallace, R. B., Johnson, M. J., Suggs, S. V., Miyoshi, K., Blatt, R., & Itakura, K. (1981) *Gene 16*, 21–26.
Wilkinson, A. J., Fersht, A. R., Blow, D. M., Carter, P., & Winter, G. (1984) *Nature (London) 307*, 187–188.
Zale, S. E., & Klibanov, A. M. (1986) *Biochemistry 25*, 5432–5444.
Zoller, M. J., & Smith, M. (1984) *DNA 3*, 479–488.

2,3-Diphosphoglycerate Phosphatase Activity of Phosphoglycerate Mutase: Stimulation by Vanadate and Phosphate†

Paul J. Stankiewicz, Michael J. Gresser,* Alan S. Tracey,* and Louis F. Hass‡
Department of Chemistry, Simon Fraser University, Burnaby, British Columbia, V5A 1S6 Canada
Received August 25, 1986; Revised Manuscript Received October 30, 1986

ABSTRACT: The binding of inorganic vanadate (V_i) to rabbit muscle phosphoglycerate mutase (PGM), studied by using ^{51}V nuclear magnetic resonance spectroscopy, shows a sigmoidal dependence on vanadate concentration with a stoichiometry of four vanadium atoms per PGM molecule at saturating [V_i]. The data are consistent with binding of one divanadate ion to each of the two subunits of PGM in a noncooperative manner with an intrinsic dissociation constant of 4×10^{-6} M. The relevance of this result to other studies which have shown that the V_i-stimulated 2,3-diphosphoglycerate (2,3-DPG) phosphatase activity of PGM has a sigmoidal dependence on [V_i] with a Hill coefficient of 2.0 is discussed. At pH 7.0, inorganic phosphate has little effect on the 2,3-DPG phosphatase activity of PGM, even at concentrations as high as 50 mM. Similarly, 25 μM V_i has little effect on the phosphatase activity. However, in the presence of 25 μM V_i, a phosphate concentration of 20 mM increases the phosphatase activity by more than 3-fold. This behavior is rationalized in terms of activation of the phosphatase activity by a phosphate/vanadate mixed anhydride. This interpretation is supported by the observation of strong activation of the phosphatase activity by inorganic pyrophosphate. A molecular mechanism for the observed effects of vanadate is proposed, and the relevance of this study to the possible use of vanadate as a therapeutic agent for the treatment of sickle cell anemia is discussed.

The 2,3-diphosphoglycerate (2,3-DPG)[1] phosphatase activity of phosphoglycerate mutase (PGM) is a well-studied phenomenon (Sasaki et al., 1971; Rose & Dube, 1978), and it has been known for some time that this activity is enhanced by inorganic vanadate, V_i (Carreras et al., 1982). This phosphatase activity has been invoked to rationalize the decrease in 2,3-DPG concentration inside erythrocytes incubated with

†Supported by grants from the Medical Research Council of Canada to M.J.G. and from the Natural Sciences and Engineering Research Council of Canada to A.S.T. and M.J.G. and by Grant HL 16647 from the National Heart, Lung, and Blood Institute to L.F.H.
*Correspondence should be addressed to this author.
‡Present address: Department of Biological Chemistry, M. S. Hershey Medical Center, Pennsylvania State University, Hershey, PA 17033.

[1] Abbreviations: 2,3-DPG, 2,3-diphosphoglycerate; PGM, phosphoglycerate mutase; V_i, inorganic vanadate; V_2, inorganic divanadate; V_4, inorganic tetravanadate; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; 2-PGA, 2-phosphoglyceric acid; 3-PGA, 3-phosphoglyceric acid.

0006-2960/87/0426-1264$01.50/0