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Stepwise Improvements in Catalytic Effectiveness: Independence and Interdependence in Combinations of Point Mutations of a Sluggish Triosephosphate Isomerase†

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ABSTRACT: Second-site suppressor changes that improve the catalytic potency of a sluggish mutant of the enzyme triosephosphate isomerase have been examined both individually and in combination. Each of the second-site mutations increases the specific catalytic activity of a triosephosphate isomerase in which the catalytic base, glutamate-165, has been changed to aspartate. These second-site suppressors are G10S, S96P, S96T, E97D, V167D, and G233R. Not one of these changes enhances the value of k_{cat}/K_m for the wild-type enzyme, which is consistent with the knowledge that the reaction catalyzed by the wild-type enzyme is already diffusion-controlled. Indeed, two of the changes, S96P and V167D, are catalytically deleterious to the wild-type isomerase. When *pairs* of second-site suppressors are combined with the primary lesion E165D, six pairs show additive independence while the effects of eight other pairs are less than additive. The sites fall into two clusters: pairs *within* a cluster always interfere with one another and do not produce additive improvements in catalytic activity, whereas combinations of changes from different clusters tend to be additive in their effects. No combination of second-site suppressor mutations behaves synergistically, though there seems to be no a priori reason to exclude this possibility. Since the catalytic potency of each of the six second-site suppressor mutants can be further improved by the introduction of (at least) one of the other five changes, it is evident that *none of the double mutants lies at a local catalytic maximum.* In these cases, therefore, the opportunity exists for at least two "steps" of monotonic catalytic improvement along each of six different "paths" in protein space.

The development of techniques for the directed and random mutagenesis of a target gene has brought some of the goals of protein engineering within reach. Manipulation of the nucleic acid sequence encoding a protein of interest permits the analysis of the contribution of any of its amino acids to stability, binding, specificity, or catalysis. Thus, proteins of increased thermal stability (Shortle & Lin, 1985; Hecht et al., 1985; Matthews et al., 1987; Pantoliano et al., 1989; Alber,

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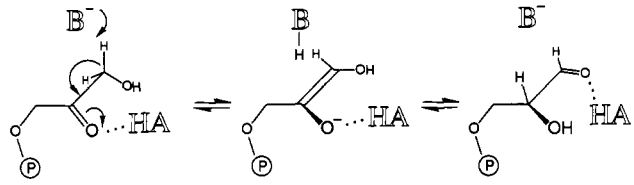
Scheme I: Interconversion of Dihydroxyacetone Phosphate and D-Glyceraldehyde 3-Phosphate, Catalyzed by Triosephosphate Isomerase



1989), enzymes of altered specificity (Carter & Wells, 1987; Wilks et al., 1988; Craik et al., 1985, 1987; Cronin & Kirsch, 1988), and enzymes of increased catalytic activity toward their natural substrates (Carter et al., 1984; Hermes et al., 1987, 1990) have all been created by the use of these methods of mutagenesis. If we are to expand our ability to produce altered proteins with desired properties, however, we must be able to predict the consequences of making multiple mutations in the protein sequence.

The work of several research groups has shown that, in most cases, multiple mutations contribute additively toward protein stability (Shortle & Lin, 1985; Matsumura et al., 1986, 1989; Nicholson et al., 1988; Stearman et al., 1988; Alber, 1989) or toward ligand (or substrate) binding [see Wells (1990) for a review]. However, analysis of the effects of combinations of changes upon enzyme catalytic activity is an area that is much less well charted. It has been suggested that most mutations that affect catalysis are additive when combined (Wells, 1990), yet work from several laboratories has shown that component mutations frequently alter the binding of the rate-limiting transition state interdependently and do not follow any simple additivity rules (Carter et al., 1984; Carter & Wells, 1988; Nagata et al., 1989; Weber et al., 1990; Hermes et al., 1990; Blacklow & Knowles, 1990; Creaser et al., 1990). In this work, we have prepared several combinations of point mutations in and near the active site of triosephosphate isomerase in order to determine for a large yet focused family of mutant enzymes the conditions under which the effects of multiple mutations on catalysis are additive.

The wild-type isomerase from chicken muscle (Figure 1) is an enzyme of subunit M_r 26 500 that catalyzes the interconversion of the glycolytic intermediates dihydroxyacetone phosphate (DHAP) and glyceraldehyde 3-phosphate (GAP), via an enediolate intermediate (Rieder & Rose, 1956, 1959; Bloom & Topper, 1958; Lodi & Knowles, 1991) (Scheme I). Inferences from the crystal structures of native isomerases (Banner et al., 1975, 1976; Lolis & Petsko, 1990) and of enzyme with bound inhibitors (Davenport, 1986; Alber et al., 1987; Lolis et al., 1990) and with bound substrate (Alber et al., 1981), combined with several other lines of evidence including affinity labeling (Hartman, 1971; de la Mare et al., 1972) and pH dependence studies (Plaut & Knowles, 1972), establish firmly that glutamate-165 is the base that abstracts the proton from substrate.

The parent enzyme for the studies reported here is the E165D mutant of triosephosphate isomerase, in which glutamate-165 has been changed to aspartate. The E165D mutant was made (Straus et al., 1985) to probe the effects of moving the catalytic carboxylate group away from the substrate by roughly 1 Å. In contrast to the wild-type enzyme, which is an extremely efficient catalyst, the rate of reaction of which is limited only by the encounter of the substrate with the active site (Albery & Knowles, 1976; Blacklow et al., 1988), the E165D enzyme has a value for k_{cat}/K_m that is roughly 250-fold lower than that of the wild type. The E165D-catalyzed reaction is no longer diffusion-controlled, but is instead limited by the chemical interconversion of the two triose phosphates (Straus et al., 1985; Raines et al., 1986). In a search for mutations at second sites that would suppress this lesion in vivo, six single-site changes in the amino acid sequence were found, each of which confers an increase in the specific catalytic activity of the protein (Hermes et al., 1989, 1990). The second-site changes were G10S, S96P, S96T, E97D, V167D, and G233R. Each of these second-site suppressor mutations lies in or near the enzyme's active site (Figure 1), and the specific catalytic activity was increased between 1.3- and 19-fold. To test whether *combinations* of these second-site suppressor mutations have an additive effect on catalysis, they were combined pairwise in all possible ways (giving a total of 14 triple mutants) with the primary lesion E165D. In addition, each of the six changes that partially suppresses E165D was introduced separately into the wild-type sequence, so that the effects on the function of the wild-type enzyme of the second-site suppressor mutations alone could be assessed.

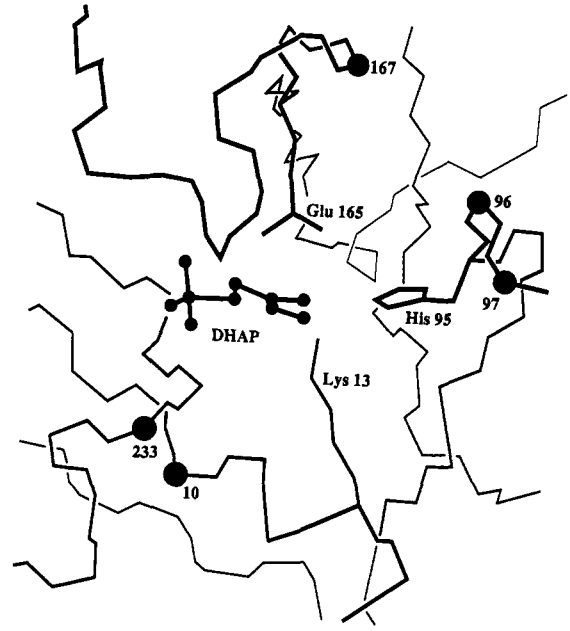


FIGURE 1: Active site of wild-type triosephosphate isomerase. The coordinates are those of the native yeast enzyme with phosphoglycolohydroxamate bound (Lolis et al., 1990). The position of the inhibitor relative to the enzyme is consistent with abstraction of the 1-*pro*-(*R*) proton of the substrate by glutamate-165, and with an electrophilic contribution to catalysis by histidine-95 and lysine-13. The Cα carbons of residues where second-site suppressor mutations occur are highlighted on the protein backbone.

Experimental Procedures

Materials. Escherichia coli strain DF 502, a streptomycin-resistant strain of *E. coli* that lacks the chromosomal triosephosphate isomerase, was a generous gift of D. Fraenkel. *E. coli* strain TG1 (Carter et al., 1985) was obtained from commercial sources. Plasmid pBSTIM, which expresses the triosephosphate isomerase from chicken muscle at about 100 mg/L of cell culture, has been described previously (Hermes et al., 1990). Mutations in the isomerase sequence are specified in brackets after the plasmid name (e.g., pBSTIM-[E165D] codes for the E165D enzyme). Reagents for DNA synthesis were purchased from Milligen/Biosearch (Burlington, MA) or from Cruachem, Inc. (Sterling, VA), except for low-water acetonitrile, which was from J. T. Baker, Inc. (Phillipsburg, NJ). Oligonucleotides for site-directed mutagenesis and for DNA sequencing were synthesized on a Milligen/Biosearch Model 7500 DNA synthesizer by using

the phosphoramidite method (Sinha et al., 1984). The restriction enzymes *Stu*I, *Pst*I, *Eco*RI, and *Hin*dIII were purchased from New England Biolabs (Beverly, MA) or from Boehringer-Mannheim Biochemicals (Indianapolis, IN). [α-^{35}S]dATP was purchased from Amersham Corp. (Arlington Heights, IL).

Triosephosphate isomerase (rabbit muscle) and glyceraldehyde-3-phosphate dehydrogenase (rabbit muscle) were obtained as suspensions in ammonium sulfate, from Boehringer-Mannheim. Traces of contaminating triosephosphate isomerase activity were removed from the coupling dehydrogenase by treatment with bromohydroxyacetone phosphate as described by de la Mare et al. (1972). Excess bromohydroxyacetone phosphate was then removed by ultrafiltration through Centricon 10 microconcentrators from Amicon (Danvers, MA) at 5000*g* (repeated twice) after dilution with 100 mM triethanolamine hydrochloride buffer, pH 7.6, containing EDTA (10 mM) at 4 °C. (*RS*)-Glyceraldehyde 3-phosphate (diethyl acetal, monobarium salt), NADH (disodium salt), and Dowex-50W (H^+ form, 100–200 mesh, 4% cross-linked) were from Sigma Chemical Co. (St. Louis, MO). The diethyl acetal of (*RS*)-glyceraldehyde 3-phosphate was hydrolyzed before use according to the manufacturer's instructions. Rich broth was prepared by using Luria Broth base from Gibco, Ltd. (Paisley, Scotland). Tris base and Tris-HCl were purchased from Sigma. KCl was AR-grade from Mallinckrodt Chemical Co. (Paris, KY). All other chemicals and reagents were of commercial reagent grade or better and were used without further purification.

Methods. pH was measured with a Corning 245 pH meter fitted with a Sigma E5634 electrode calibrated at room temperature with Fisher standard buffers. Ultraviolet and visible absorbance measurements were made on a Hewlett-Packard 8452A diode array spectrophotometer thermostated with a Brinkmann RMS 6 temperature controller. The Amersham oligonucleotide-directed mutagenesis system was used for the preparation of site-directed mutations. DNA sequencing was performed with a Sequenase version 2.0 kit from United States Biochemical Corp. (Cleveland, OH). Unless otherwise noted, manipulations of DNA were performed as described in Sambrook et al. (1989). Ion-exchange chromatography for the purification of mutant enzymes was performed on an FPLC system from Pharmacia (Piscataway, NJ), consisting of two P-500 high-precision pumps, a UV-M absorbance monitor, an REC two-channel recorder, and a V7 valve injection port. Chromatography fractions (1.5 mL) were collected on a Pharmacia FRAC-100 fraction collector.

Construction of Mutant-Encoding Plasmids. To incorporate the desired point mutations into the isomerase gene, the Amersham oligonucleotide-directed mutagenesis system, version 2.0, which is based on the method of Eckstein and co-workers (Taylor et al., 1985; Nakamaye & Eckstein, 1986; Sayers et al., 1988), was used. Synthetic oligonucleotides of between 18 and 24 bases were designed to introduce the G10S, S96P, S96T, E97D, V167D, and G233R changes into the wild-type sequence of the chicken isomerase gene. Single-stranded template of the wild-type pBSTIM phagemid was prepared as recommended (Taylor et al., 1985). After the mutagenesis procedure was carried out, colonies harboring plasmids that contained the desired mutations were identified by dideoxy sequencing of the plasmid DNA (Sanger et al., 1977; Tabor & Richardson, 1987), using a Sequenase kit.

Construction of Plasmids Expressing Isomerases with Multiple Mutations. Of the 14 triple mutants, 9 were constructed by restriction enzyme cleavage of plasmids encoding mutant isomerases containing the parent E165D mutation and a suppressor mutation, followed by ligation of the appropriate purified fragments to produce genes containing E165D and two suppressor mutations. Three restriction enzymes were used: *Hin*dIII and *Stu*I, which cut at positions 170 and 360 within the gene, respectively, and *Pst*I, which cuts 293 base pairs beyond the 3′ end of the gene. In this way, [E165D, G10S, S96P], [E165D, G10S, S96T], and [E165D, G10S, E97D] were constructed by digestion of the plasmid DNA with the restriction enzymes *Hin*dIII and *Pst*I, followed by ligation of appropriate fragments with T4 DNA ligase. The [E165D, G10S, V176D], [E165D, G10S, G233R], [E165D, S96P, G233R], [E165D, S96T, G233R], [E165D, E97D, V167D], and [E165D, E97D, G233R] triple mutants were constructed in a similar fashion using the restriction enzymes *Stu*I and *Pst*I.

The other five triple mutants were constructed by oligonucleotide-directed mutagenesis using the Amersham oligonucleotide-directed mutagenesis system, as described above. The [E165D, S96P, V167D], [S96T, E165D, V167D], and [E165D, V167D, G233R] proteins were made by using a pBSTIM[E165D, V167D] template and oligonucleotides encoding the S96P, S96T, and G233R changes, respectively. The [E165D, S96P, E97D] and [E165D, S96T, E97D] proteins were constructed by using a pBSTIM[E165D] template and oligonucleotides encoding the two pairs of changes, S96P, E97D and S96T,E97D, respectively.

Purification of the Mutant Isomerases. Mutant isomerases were purified by a method described previously (Hermes et al., 1989). *E. coli* DF502 cells were transformed with the appropriate plasmid, and the cells were plated onto rich plates supplemented with ampicillin (sodium salt, 200 mg/L) and streptomycin (sulfate salt, 100 mg/L) to select for transformants. Colonies from these plates were grown overnight in Luria broth (5 mL), supplemented with ampicillin (200 mg/L) and streptomycin (100 mg/L). A portion of each culture (1 mL) was used to inoculate a flask of Luria broth [100 mL, supplemented with ampicillin (200 mg/L) and streptomycin (100 mg/L)], and the culture was grown for 24 h. The cells were harvested by centrifugation (5000*g*) at 4 °C. The cell paste was resuspended in 5 mL of 10 mM Tris-HCl buffer, pH 8.0, and the cells were lysed by passing the cell suspension through an Aminco (Urbana, IL) French pressure cell at 20 000 psi. The resulting lysate was cleared by ultracentrifugation for 2 h at 150000*g*. The recovered supernatant was concentrated in a Centricon 10 ultrafiltration microconcentrator at 4 °C. After dilution with 10 mM Tris-HCl buffer, pH 8.0, the solution was again concentrated at 5000*g*; this step was repeated once more. The concentrated solution was passed through a 0.22-μm filter (Gelman Sciences, Ann Arbor, MI), and the filtrate was subjected to ion-exchange chromatography on a column of Mono Q HR 5/5 resin (Pharmacia). The mutant isomerase was eluted with a linear gradient of KCl (0–150 mM) in 10 mM Tris-HCl buffer, pH 8.0. In each case, the isomerase was collected as a single peak as determined by A_{280nm}, and the enzyme preparation was homogeneous as assessed by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate (Laemmli, 1970).

Steady-State Kinetics. Enzyme assays were based on the method of Putman et al. (1972), at 30 °C in 100 mM triethanolamine hydrochloride buffer, pH 7.6, containing EDTA (10 mM). Mutant enzymes were assayed by using at least four different concentrations of glyceraldehyde 3-phosphate. The extinction coefficient of NADH was taken to be 6220 M^{-1} cm^{-1} at 340 nm (Horecker & Kornberg, 1948). The validity

of the assays was checked by ensuring the linear dependence of the observed initial rate on the concentration of triosephosphate isomerase at both high and low substrate concentrations. The values of k_{cat} and K_m for each substrate were determined by using the computer program HYPERO (Cleland, 1979).

Results and Discussion

In the work reported here, we have analyzed the catalytic behavior of a family of mutants of the enzyme triosephosphate isomerase. This group of altered enzymes is comprised of mutants containing single and multiple changes in the region of the active site. The impact of the individual amino acid alterations on the catalytic activity of the wild-type enzyme has been assessed by analysis of each of the point mutants, and the interaction between pairs of sites has been investigated by study of mutant enzymes in which component mutations have been combined. Specifically, we have measured the effect of six suppressor mutations of the catalytically deficient E165D mutant of the enzyme, both when introduced alone into the wild-type sequence and when combined pairwise in the context of E165D.

The E165D enzyme was originally constructed to investigate the importance of the precise placement of the catalytic base at the active site. The conservative substitution of glutamate-165 by aspartate is responsible for a dramatic decrease in catalytic effectiveness, such that the value of k_{cat} for the mutant is decreased about 1000-fold relative to the wild type. Whereas the rate of the reaction catalyzed by the wild-type enzyme is diffusion-controlled, the rate of the reaction mediated by the E165D mutant enzyme is limited by the enolization of substrate dihydroxyacetone phosphate. The observed decrease in activity of the mutant enzyme is not due to a change in chemical mechanism (Raines et al., 1986), nor does it derive from substantial structural effects that are propagated to other regions of the protein. Difference electron density maps of the wild-type and mutant enzymes show that the only significant change in the mutant enzyme is a movement of the catalytic carboxylate group of about 1 Å (E. Lolis and G. A. Petsko, personal communication).

To discover whether the catalytic activity of the E165D enzyme could be increased by compensating mutations elsewhere in the protein, the whole of the gene encoding the E165D isomerse was subjected to an efficient procedure for random mutagenesis, and isomerases of improved catalytic potency were selected (Hermes et al., 1989). Six amino acid changes at five second sites increased the specific catalytic activity of the E165D enzyme: G10S, S96P, S96T, E97D, V167D, and G233R (Hermes et al., 1990). While a complete interpretation of the effect of each of these suppressor mutations upon the parent E165D enzyme awaits structural analysis, each of the changes lies within the region of the active site, and each involves a residue that is highly conserved among the dozen species for which the sequence of the isomerase gene is known.

To determine the effect of the suppressor mutations upon catalysis by the wild-type enzyme, each of the six changes G10S, S96P, S96T, E97D, V167D, and G233R was introduced individually into an otherwise wild-type sequence by site-directed mutagenesis. The kinetic parameters k_{cat} and K_m for glyceraldehyde 3-phosphate as substrate were determined for each of the six mutant isomerases, and these data are reported in Table I. The kinetic parameters for each of the six double mutants ([E165D, G10S], [E165D, S96P], [E165D, S96T], [E165D, E97D], [E165D, V167D], and [E165D, G233R]) are reproduced in Table II.

Table I: Kinetic Characteristics of Seven Single Mutants of Triosephosphate Isomerase[a]

enzyme[b]	$\vec{k}_{cat}$ (s^{-1})	$\vec{K}_m$ (mM)	$\vec{k}_{cat}/\vec{K}_m$ ($mM\ s^{-1}$)	$\vec{k}_{cat}$ (relative)	$\vec{k}_{cat}/\vec{K}_m$ (relative)
wild type	3500	0.47	7500	1.0	1.0
G10S	1700	0.52	3300	0.48	0.45
S96P[c]	64	0.087	740	0.018	0.098
S96T	1100	0.19	5700	0.31	0.76
E97D	3600	0.30	12100	1.0	1.6
E165D	4.3	0.080	54	0.0012	0.0072
V167D	58	0.033	1700	0.016	0.23
G233R	1650	0.23	7200	0.47	0.96

[a] The superscript arrow pointing to the left signifies that glyceraldehyde 3-phosphate was used as the substrate. [b] Designations use the single-letter amino acid code, such that G10S signifies an enzyme in which glycine-10 has been changed to serine. [c] Blacklow and Knowles (1990).

Table II: Kinetic Characteristics of the Starting Mutant Triosephosphate Isomerase and the Six Pseudorevertant Mutants[a,b]

enzyme[b]	$\vec{k}_{cat}$ (s^{-1})	$\vec{K}_m$ (mM)	$\vec{k}_{cat}/\vec{K}_m$ ($mM\ s^{-1}$)	$\vec{k}_{cat}$ (relative)	$\vec{k}_{cat}/\vec{K}_m$ (relative)
E165D	4.3	0.080	54	1.0	1.0
E165D, G10S	14	0.18	78	3.3	1.4
E165D, S96P	68	0.066	1000	16	19
E165D, S96T	17	0.10	170	4.0	3.1
E165D, E97D	10	0.079	130	2.4	2.3
E165D, V167D	15	0.17	88	3.6	1.6
E165D, G233R	8.4	0.029	290	2.0	5.4

[a] The superscript arrow pointing to the left signifies that glyceraldehyde 3-phosphate was used as the substrate. [b] Hermes et al. (1990). [c] Designations use the single-letter amino acid code, such that G10S signifies an enzyme in which glycine-10 has been changed to serine.

Theory. Individual mutations in a protein can act either independently or interactively in a double mutant. If the free energy change in the measured property due to the first mutation is denoted by $\Delta\Delta G_1$, and that due to the second mutation is $\Delta\Delta G_2$, the change in free energy observed in the double mutant, $\Delta\Delta G_{1,2}$, can be expressed as (Carter et al., 1984; Ackers & Smith, 1985; Wells, 1990)

$$\Delta\Delta G_{1,2} = \Delta\Delta G_1 + \Delta\Delta G_2 + \Delta G_i \quad (1)$$

where ΔG_i represents an interaction energy between sites. When ΔG_i is near or equal to zero, the sites behave independently, and the double mutant displays "simple additivity" (Wells, 1990) with respect to the two point mutations. When ΔG_i differs significantly from zero, the mutations are interactive in the double mutant. If the functional property is enhanced in the double mutant relative to that predicted from simple additivity, then the mutations act synergistically; when the property is diminished relative to the predicted value, then the mutations behave antagonistically.

Application to Triosephosphate Isomerase: Single and Double Mutants. The rate-limiting transition state of the reaction catalyzed by wild-type triosephosphate isomerase involves the diffusion of substrate glyceraldehyde 3-phosphate to the enzyme's active site (Albery & Knowles, 1976; Blacklow et al., 1988). Consequently, mutations that accelerate any of the chemical (bond-making or bond-breaking) steps in the overall conversion of substrate to product will have little or no effect upon the measured rate of the reaction. Mutations in triosephosphate isomerase that affect the rates of the

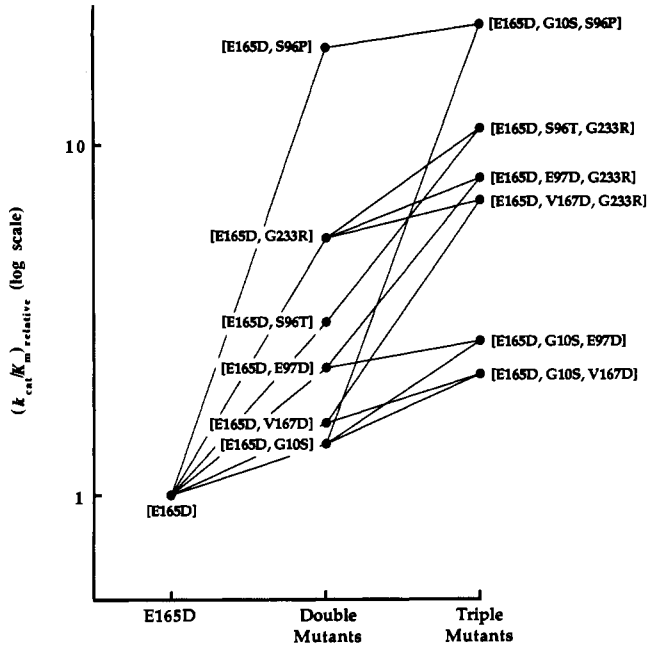


FIGURE 2: Values of k_{cat}/K_m for triple mutants obtained by combining two of the second-site suppressor mutations with the parent E165D mutation. Mutants where the effects are additive are illustrated.

chemical steps in the catalyzed reaction will deviate from simple additivity if one of the mutations slows the rate of a chemical step so that it becomes rate-limiting in the mutant, and the other mutation acts to accelerate the rate of that step in the wild-type enzyme. In the wild-type context, the effect of the second change will be masked because it increases the rate of a step that is already kinetically insignificant.

The data presented in Tables I and II are consistent with this simple analysis. Mutations that partially suppress the sluggishness of the E165D mutant accelerate the chemical step that is rate-limiting for the E165D isomerase. Because the wild-type enzyme is already diffusion-limited, no dramatic increases in the catalytic efficiency of the wild-type enzyme were expected when each suppressor mutation was separately introduced into the wild-type context. Indeed, none of these mutants shows a significant increase in the value of k_{cat}/K_m for glyceraldehyde 3-phosphate (Table I). Four of the six enzymes, G10S, S96T, E97D, and G233R, are not significantly different from the wild type in their kinetic characteristics (Table I).

Two of the component mutations, S96P and V167D, each of which causes an increase in specific catalytic activity when inserted into the E165D isomerase, are deleterious when introduced into the wild-type sequence. The values of k_{cat} for S96P and for V167D are decreased about 50- and 60-fold, respectively, from wild type (Table I). It is likely that each of these changes disrupts the precise arrangement of catalytic groups at the active site, causing the chemical interconversion of substrate and product to become rate-limiting. The V167D mutation introduces a new negative charge approximately 9 Å from the catalytic base, glutamate-165. Electrostatic repulsion between these negatively charged side chains or movement of the main chain atoms of residue 165 consequent upon the V167D change, or both, could easily displace the carboxylate of E165. Similarly, replacement of serine-96 by proline may affect the positioning of histidine-95, a residue that is known to play a critical electrophilic role in catalysis by the wild-type enzyme (Belasco & Knowles, 1980; Nickbarg et al., 1988; Blacklow & Knowles, 1990; Komives et al., 1991). However, whatever the reason for the deleterious effects of the S96P and V167D mutations on catalysis by the wild-type enzyme, the fact that each of these changes *improves* the catalytic potency of the E165D enzyme demonstrates an interdependence in the two pairs of loci (165 and 167; 165 and 96) on the chemical steps that are rate-limiting for each mutant.

Table III: Kinetic Characteristics of the Starting Mutant Triosephosphate Isomerase and the 14 Triple Mutants[a]

enzyme[b]	$\vec{k}_{cat}$ (s^{-1})	$\vec{K}_m$ (mM)	$\vec{k}_{cat}/\vec{K}_m$ ($mM\ s^{-1}$)	$\vec{k}_{cat}$ (relative)	$\vec{k}_{cat}/\vec{K}_m$ (relative)
E165D	4.3	0.080	54	1.0	1.0
E165D, G10S, S96P	67	0.056	1200	16	22.2
E165D, G10S, S96T	34	0.26	130	8.1	2.4
E165D, G10S, E97D	25	0.17	150	6.0	2.74
E165D, G10S, V167D	28	0.24	120	6.7	2.2
E165D, G10S, G233R	24	0.083	280	5.6	5.2
E165D, S96P, G233R[c]					
E165D, S96T, G233R	30	0.05	600	7.2	11.1
E165D, E97D, V167D	19	0.31	61	4.5	1.1
E165D, E97D, G233R	18	0.042	430	4.3	8.0
E165D, S96P, E97D	40	0.081	500	9.3	9.2
E165D, S96P, V167D	34	0.51	66	14	1.2
E165D, S96T, E97D	17	0.60	28	3.8	0.51
E165D, S96T, V167D	12	0.24	49	2.7	0.9
E165D, V167D, G233R	29	0.076	380	6.6	6.9

[a] The superscript arrow pointing to the left signifies that glyceraldehyde 3-phosphate was used as the substrate. [b] Designations use the single-letter amino acid code, such that G10S signifies an enzyme in which glycine-10 has been changed to serine. [c] Mutant displayed nonlinear kinetics.

Isomerase Mutants in Which E165D Is Combined with Two Suppressor Mutations. Each of 14 possible isomerases in which 2 of the suppressor mutations are combined with E165D has been constructed. The kinetic parameters k_{cat} and K_m were determined for each of these triple mutants with glyceraldehyde 3-phosphate as substrate, and these values are reported in Table III. The triple mutants fall into two groups. In the first group, the effects of the two individual suppressor mutations combine additively in the triple mutant. These enzymes are [E165D, G10S, S96P], [E165D, G10S, E97D], [E165D, G10S, V167D], [E165D, S96T, G233R], [E165D, E97D, G233R], and [E165D, V167D, G233R], and the additivity of the kinetic effects is illustrated by the existence of the parallelograms shown in Figure 2. Significantly, in each of these cases, the two suppressor mutant loci are not close

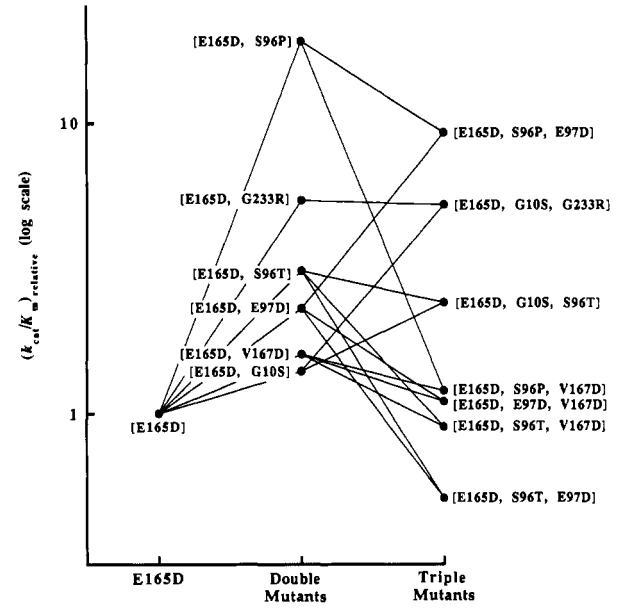


FIGURE 3: Values of k_{cat}/K_m for triple mutants obtained by combining two of the second-site suppressor mutations with the parent E165D mutation. Mutants where the effects are nonadditive are illustrated.

to one another in the active site: each pair appears to fulfill the criterion of Wells (1990) for simple site additivity.

The second group of enzymes with multiple site changes comprises those in which the effects of the two suppressor mutations are less than additive when combined. These enzymes are [E165D, G10S, S96T], [E165D, G10S, G233R], [E165D, S96P, E97D], [E165D, S96T, E97D], [E165D, S96P, V167D], [E165D, S96T, V167D], [E165D, E97D, V167D], and [E165D, S96P, G233R],[1] and the nonadditivity of the effects of these changes is illustrated by the quadrilaterals shown in Figure 3. For all but two of these triple mutants, site interdependence can be rationalized in terms of local or specific interactions that are expected when residues that are contiguous or are close to one another are changed simultaneously. These clusters fall into two groups: the first comprises residues 96, 97, and 167, and the second consists of residues 10 and 233. Neither of the enzymes in which residues 96 and 97 are altered together combine additively: ΔG_i is significantly greater than zero for both the [E165D, S96P, E97D] and [E165D, S96T, E97D] enzymes. Similarly, the peptide backbone in the region of glycine-233 approaches within 8 Å of that of glycine-10, and it is reasonable to suppose that the side chains of serine-10 and arginine-233 interfere with each other in the triple mutant [E165D, G10S, G233R].

Of the five other triple mutants that fail to show site additivity at the suppressor loci, three combine V167D with mutations at positions 96 or 97. This interdependence seems likely to arise from the interactions that are known to exist between the two regions of the protein spanned by these residues. In the crystal structure of wild-type isomerase from yeast, the side-chain hydroxyl group of serine-96 forms a hydrogen bond with the carboxylate of the catalytic base, glutamate-165 (Lolis et al., 1990). Serine-96 (and by extension, the adjacent residue glutamate-97) is coupled to glutamate-165 through this hydrogen-bonding interaction. It is therefore not surprising that the V167D mutation, which is two residues away from glutamate-165, does not follow simple additivity when combined with any of the changes at positions 96 and 97. In addition, the side chain of valine-167 lies only 4 Å from the side chain of serine-96, and the presence of a new negative charge consequent upon the V167D mutation seems likely to affect the positions of the side chains of serine-96 and glutamate-97 directly. Antagonism between the G10S and S96T mutations and between the S96P and G233R mutations is less easily explained, and interpretation of the kinetic consequences of these multiple changes (in the [E165D, G10S, S96T] and [E165D, S96P, G233R] mutants) must await more detailed structural information.

Finally, it may be noted that we have found no examples of pairs of nonadditive changes that are synergistic rather than antagonistic. There would seem to be no a priori arguments against this possibility, and we presume that the very limited set of triple mutants that we have examined here may simply not include examples of such synergistic effects. Starting from each of the double mutants, we have only made 4 or 5 specific changes, and have thus scanned an insignificantly small proportion of all possible third-site changes, which for this enzyme amount to 4674 (19 alternative amino acids at 246 sites). A more thorough search will require, for example, the application of the random mutagenesis protocol that generated our six second-site suppressors in the first place (Hermes et al., 1990).

Summary and Conclusions. In the work reported here, we have examined the kinetic behavior of 27 mutants of the enzyme triosephosphate isomerase. Seven of these mutants contain amino acid changes at single sites, and the rest have mutations at two or three sites. Of the 20 enzymes containing multiple mutations, 10 clearly fail to show simple additivity in the effects of the mutations at the individual sites. It has been suggested earlier that combinations of mutations will show simple additivity unless specific interactions prohibit the sites from functioning independently (Wells, 1990). We believe that these conditions are necessary, but probably not sufficient, to produce simple site additivity, for there are mutants of triosephosphate isomerase for which simple additivity would certainly have been anticipated, but is not found (e.g., for the [E165D, G10S, S96T] and [E165D, S96P, G233R] mutants).

The experiments described also demonstrate an important (though still controversial) feature of protein sequence "space" (Maynard-Smith, 1970). Thus, our results show that it is possible to improve the catalytic effectiveness of an enzyme *incrementally* by combining mutations that individually enhance the rate of the reaction. The landscape of sequence space is not particularly rugged, and the effects of individual mutations may be cumulative in many cases. Not one of the six double mutants that derived from our search for second-site suppressors turns out to lie at a local maximum of catalytic efficiency. Even the [E165D, S96P] enzyme, which has the highest specific catalytic activity of all the double mutants, can be further improved by the addition of the G10S change (see Tables II and III). These results must further encourage those who aim to engineer enzymes with new or improved catalytic activities, for our data suggest that strategies for protein engineering that employ combinations of mutations in an incremental way to provide the desired property stand a reasonable likelihood of success.

[1] The mutant enzyme containing the amino acid changes [E165D, S96P, G233R] did not follow linear Michaelis–Menten kinetics, and values for k_{cat} and for K_m were not determined for this enzyme.

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