Electrophilic Assistance by Asp-99 of 3-Oxo-Δ^5-steroid Isomerase†

Lora D. Thornburg,‡ Frédéric Hénot,‡ Diarmad P. Bash,‡ David C. Hawkinson,§ Shawn D. Bartel,§ and Ralph M. Pollack*,‡,||

Laboratory for Chemical Dynamics, Department of Chemistry and Biochemistry, 1000 Hilltop Circle, University of Maryland, Baltimore County, Baltimore, Maryland 21250, Center for Advanced Research in Biotechnology, 9600 Gudelsky Drive, Rockville, Maryland 20850, and Department of Chemistry, University of South Dakota, Vermillion, South Dakota 57069

Received January 13, 1998; Revised Manuscript Received May 4, 1998

ABSTRACT: 3-Oxo-Δ^5-steroid isomerase (Δ^5-3-ketosteroid isomerase, KSI; EC 5.3.3.1) catalyzes the conversion of a variety of β,γ-unsaturated 3-oxosteroids to their corresponding α,β-unsaturated isomers at rates that approach the diffusion limit for specific substrates. The reaction proceeds through a dienolate intermediate, with two amino acid residues (Asp-38 and Tyr-14) known to be involved in catalysis. When the complete three-dimensional structure of KSI was determined recently by NMR methods, an additional polar residue (Asp-99) was found in the active site and this group was shown to be important for catalytic activity. In this work, we examine the properties of several mutant KSIs to determine the nature of catalysis by Asp-99 of KSI. The electrophoretic mobilities of wild-type (WT) KSI and several mutants (D99A, D99N, D38N, and D38N/D99A) on native gels were determined at pH values ranging from 6.0 to 8.5. The results demonstrate that the pK_a of Asp-99 is >8.5 in wild-type KSI. The pH−rate profiles for the D99A, D99N, and D38H/D99A mutants of KSI were also determined. For all three mutants, k_{cat} and k_{cat}/K_M do not decrease at high pH, in contrast to those for WT and D38H, which lose activity above pH 9 and 8, respectively. Mutation of Asp-99 to Asn decreases k_{cat} for the substrate 5-androstene-3,17-dione by 27-fold and k_{cat}/K_m by 23-fold, substantially less than the loss of activity (3000-fold in k_{cat} and 2200-fold in k_{cat}/K_m) observed when Asp-99 is mutated to Ala, consistent with a hydrogen bonding role for Asp-99. Taken together, these results provide evidence that Asp-99 participates in catalysis in its protonated form, with a pK_a of >9 in WT and ∼8.5 in the D38H mutant. Asp-99 likely donates a hydrogen bond to O-3 of the steroid, helping to stabilize the transition state(s) of the KSI-catalyzed reaction.

The nature of enzymatic catalysis of the cleavage of a C−H bond adjacent to a carbonyl group to generate an enolate ion or enol has become a topic of widespread research activity. Since aldehydes and ketones are generally only moderately acidic (pK_as > 12) and breaking C−H bonds is usually a slow process (*1*), the mechanism by which enzymes stabilize the enol(ate) and the flanking transition states is of fundamental interest. It has been proposed that the catalytic activity of these enzymes can be accounted for by the formation of low-barrier hydrogen bonds between an enzyme electrophile and the oxygen of the incipient enolate ions (*2*−*5*), but this hypothesis is not without its critics (*6*−*9*). Alternatively, stabilization of intermediates and transition states may be accomplished by multiple interactions of moderate strength, as demonstrated by Shan and Herschlag in model systems (*10*).

A particularly well-studied example of enzymatic enolization is the reaction catalyzed by 3-oxo-Δ^5-steroid isomerase (Δ^5-3-ketosteroid isomerase, KSI;[1] EC 5.3.3.1). This enzyme converts 3-oxo-Δ^5-steroids such as **1** to their conjugated Δ^4-isomers (**3**) by sequential enolization and ketonization reactions (Scheme 1) at rates that approach the diffusion limit for specific substrates (*11*). It has been generally accepted for some time that Asp-38 acts as a base to abstract a proton from C-4 and subsequently return it to C-6, while Tyr-14 stabilizes the intermediate and transition states by hydrogen bonding with O-3 of the steroid (*12*−*16*). A comparison of the free energy profiles for conversion of **1** to **3** by KSI and by acetate ion shows that the enzyme-bound dienolate intermediate (**2**) and transition states are each stabilized by ∼10 kcal/mol (*11*, *17*).

Recently, Wu et al. (*18*) determined the complete three-dimensional structure of KSI from *Pseudomonas testosteroni* by NMR methods and found an additional polar residue (Asp-99) in the active site. On the basis of its position relative to Asp-38, Tyr-14, and a computationally docked

† This work was supported by Grant GM 38155 from the National Institutes of Health.

* Address correspondence to this author at University of Maryland, Baltimore County.

‡ University of Maryland, Baltimore County.

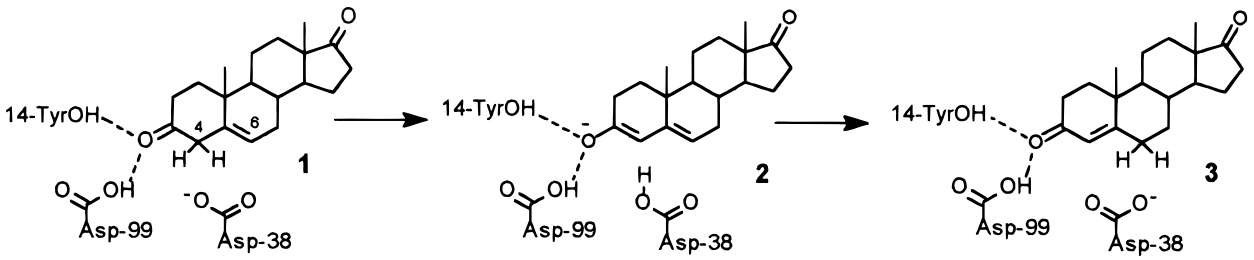
§ University of South Dakota.

|| Center for Advanced Research in Biotechnology.

[1] Abbreviations: ADA, *N*-(2-acetamido)-2-iminodiacetic acid; BSA, bovine serum albumin; CAPS, 3-(cyclohexylamino)-1-propanesulfonic acid; CHES, 2-(cyclohexylamino)ethanesulfonic acid; DEAE, diethylaminoethyl; IPTG, isopropyl β-D-thiogalactopyranoside; K_E, acid dissociation constant obtained from the k_{cat}/K_m versus pH plot; K_{ES}, acid dissociation constant obtained from the k_{cat} versus pH plot; KSI, 3-oxo-Δ^5-steroid isomerase (EC 5.3.3.1); MES, 2-(*N*-morpholino)ethanesulfonic acid; PAGE, polyacrylamide gel electrophoresis; PEG, poly(ethylene glycol); RF-DNA, replicative form of M13 DNA; SDS, sodium dodecyl sulfate; TES, *N*-[tris(hydroxymethyl)methyl]-2-aminoethanesulfonic acid; Tris, tris(hydroxymethyl)aminomethane; WT, wild-type KSI from *P. testosteroni*.

S0006-2960(98)00099-3 CCC: $15.00
Published on Web 06/26/1998

Scheme 1



substrate molecule, Asp-99 was postulated to form a second hydrogen bond with the intermediate and transition states (Scheme 1). Thus, two hydrogen bonds of moderate strength, rather than a single strong hydrogen bond from Tyr-14, would contribute to catalysis. Consistent with this hypothesis is the observation that the D99A mutant, in which Asp-99 is replaced by Ala, is $>10^3$-fold less active than wild-type (WT) KSI (*18*). In addition, Asp-99 is conserved in the KSI from *Pseudomonas putida*,[2] which is 34% identical in sequence to *P. testosteroni* KSI (*19*), as well as in the KSI from *Nocardioides simplex* (*20*).[2,3] Further support for the mechanism of Scheme 1 is provided by the X-ray structure of the KSI from *P. putida* with the intermediate analogue equilenin bound in the active site (*21*). This structure, which is analogous to the solution structure of the *P. testosteroni* enzyme, shows that the oxygen of Tyr-14 and one of the carboxyl oxygens of Asp-99 are both within hydrogen bonding distance of O-3 of the steroid. An alternate mechanism, involving hydrogen bond donation from the COOH of Asp-99 to the OH of Tyr-14, has also been proposed to account for the catalytic contribution of Asp-99 (*22*).

The contribution of Asp-99 to the catalytic mechanism of KSI offers an explanation for previous work that implicates a group with a pK_a of >9 in the mechanism of action of KSI. The pH−rate profile for WT was first determined in 1970 by Weintraub et al. (*23*), who observed maximum V_{max} values for the specific substrate 5-androstene-3,17-dione between pH 7 and 9. Below that range, enzymatic activity decreases with an apparent pK_a of 5.6. V_{max} also decreases at high pH, with an apparent pK_a of 9.3; at pH 10, it is only 20% of the maximum value. In subsequent work with the nonsticky substrate 5(10)-estrene-3,17-dione, we observed that both k_{cat} and k_{cat}/K_m decrease at low pH, with a dependence on a group with pK_as of 4.7 and 4.6, respectively, but that the enzyme is irreversibly inactivated above pH 9 (*24*). More recently, Holman and Benisek (*25*) obtained a bell-shaped pH−rate profile for k_{cat}/K_M of the D38H KSI mutant, which gave pK_a values of 4.4 and 8.5. They attributed the former to His-38, which might act as the catalytic base replacing Asp-38, but the group being titrated at pH 8.5 could not be identified, although UV titration ruled out a tyrosine residue. The most straightforward interpretation of these results is that Asp-99 has a perturbed pK_a of >9 in WT and 8.5 in D38H and that it is the group responsible for the high-pH behavior of both enzymes.

[2] *P. testosteroni* numbering is used for the *P. putida* and *N. simplex* enzymes.

[3] Y. Murooka (1994), direct submission of the *N. simplex* sequence to the DDBJ/EMBL/GenBank databases.

In this work, we demonstrate that, in agreement with prediction, Asp-99 *does* have an unusually high pK_a for a carboxylic acid, enabling it to participate as a hydrogen bond donor in catalysis. In addition, we find that replacement of Asp-99 with Asn (D99N) results in an enzyme that is intermediate in activity between WT and the D99A mutant, consistent with a hydrogen bonding role for this residue. We also demonstrate that Asp-99 is responsible for the loss in activity at high pH for both WT and D38H by determining the pH−rate profiles for the D99A, D99N, and D38H/D99A mutants.

EXPERIMENTAL PROCEDURES

Materials. D38N, WT, and 5-androstene-3,17-dione (**1**) were available from previous work (*17*, *26*, *27*). Oligonucleotide primers were obtained from the Biopolymer Laboratory at the University of Maryland, Baltimore. The Sculptor in vitro mutagenesis kit was purchased from Amersham Life Science Inc. The QuikChange Site-Directed Mutagenesis Kit was supplied by Stratagene. T4 DNA ligase, *Eco*RI, *Hin*dIII, and the Wizard Plus Miniprep DNA Purification system were supplied by Promega Corp. *Bam*HI and *Pvu*I were purchased from Life Technologies Inc. (GibcoBRL). *Mbo*II was purchased from New England Biolabs. DEAE-Sephacel and equilenin were purchased from Sigma. BSA was obtained from Calbiochem and used without purification. Other reagents were reagent grade or better and are commercially available. Water was purified by reverse osmosis.

General Methods. Molecular biology protocols were performed as described by Sambrook et al. (*28*) unless otherwise noted. Liquid bacterial cultures were grown on an orbital shaker at 200−250 rpm. M13 single-stranded DNA was purified by PEG precipitation and phenol extraction. Plasmid DNA and M13 RF-DNA were isolated using the Plasmid Midi Kit from QIAGEN Inc. Restriction enzyme and T4 DNA ligase reactions were carried out according to the manufacturers' instructions. DNA restriction fragments were isolated using an agarose gel DNA extraction kit purchased from Boehringer Mannheim Corp. Polyacrylamide gel electrophoresis was carried out using a Bio-Rad Mini-PROTEAN II apparatus. UV spectra and kinetic data were acquired on a Gilford Response I or Response II or Cary 1 Bio spectrophotometer equipped with thermostated sample blocks. Stock solutions of **1** in methanol were prepared fresh each day. The concentration of **1** was determined from the absorbance at 248 nm after complete conversion to **3** (ϵ = 16 300 M^{-1} cm^{-1}; *29*) by WT. Binding of equilenin to D38N and D38N/D99A was measured by fluorescence quenching, as previously described

(*17*). Experimental data were fit to equations using a least-squares program based on the Marquardt algorithm.

Mutagenesis and Expression Plasmid Construction for D99A, D99N, and D38N/D99A. Site-directed mutagenesis was performed using Amersham's Sculptor kit, which is based on the phosphorothiolate method of Eckstein and co-workers (*30*−*32*). The template used to prepare the single mutants D99A and D99N contained the wild-type *P. testosteroni* KSI gene (375 bp) on a 1.2 kb fragment inserted into the *Pst*I site of M13mp8 (*33*). The template used for the double mutant D38N/D99A was M13mp8 containing the D38N KSI mutant gene previously prepared by site-directed mutagenesis (*17*). The Asp-99 to Asn mutation was created with the primer 5′-GCGAAAGTGATTGATGGGCGC-3′. The Asp-99 to Ala mutation, as well as a silent mutation at Pro-97 that generated a *Pvu*I restriction site, was created with the primer 5′-GCGAAAGTGAGCGATCGGCGCAACC-3′ (mismatched bases are underlined). Mutant clones were identified by two-base sequencing of single-stranded DNA in the case of D99N and by restriction digestion of RF-DNA by *Pvu*I in the cases of the D99A and D38N/D99A mutants. To verify that only the desired mutation was present, the entire KSI gene of each mutant was sequenced by the Biopolymer Laboratory at the University of Maryland, Baltimore. *Bam*HI−*Hin*dIII fragments (1.2 kb) containing the mutant KSI genes were isolated from RF-DNA and ligated into pUC18 to create the expression plasmids pLDT40 (D99A), pLDT41 (D99N), and pLDT47 (D38N/D99A).

Mutagenesis and Expression Plasmid Construction for D38H/D99A. The D38H/D99A mutant of KSI was prepared using the QuikChange Site-Directed Mutagenesis Kit, which employs Pfu polymerase and a thermocycler (Powerblock System, Ericomp). The pLDT40 vector was used with two primers 5′-GGAACCCACGGGGTGTTCCACCGTGG-3′ and 5′-CCACGGTGGAACACCCCGTGGGTTCC-3′ designed to introduce the D38H mutation into the D99A KSI gene. Recombinant plasmids were transformed into Epicurian Coli XL1-Blue supercompetent cells (Stratagene) and purified from the transformants by using the Wizard Plus Miniprep DNA Purification system. Since the D38H mutation eliminates the *Mbo*II site at position 109 in the KSI gene, suitable mutants were selected by enzymatic digestion with *Mbo*II and analysis of the resulting DNA fragments on a 2% agarose gel. The entire KSI gene in positive clones was then sequenced by the Biopolymer Laboratory at the University of Maryland, Baltimore.

Protein Expression and Purification. For D99A, D99N, and D38N/D99A, expression plasmids were transformed into DH5α *Escherichia coli*. Liquid cultures (2 L per 6 L flask) were grown at 37 °C in LB medium containing 100 μg/mL ampicillin and 0.5 mM IPTG. Cells were harvested by centrifugation after growth for 12−16 h. D38H/D99A was expressed in Epicurian Coli XL1-Blue cells by inoculation of 6 L of 2YT medium (16 g of bactopeptone, 10 g of yeast extract, and 5 g of NaCl per liter) supplemented with 0.76 mM IPTG and 100 μg/mL ampicillin, with 300 mL of late log phase culture. Incubation was performed for 16 h at 37 °C while the mixture was shaken constantly.

The enzyme purification, summarized below, is based on a combination of published procedures (*29*, *34*). The harvested cells were resuspended in 50 mM Tris-HCl (pH 7.5), disrupted by sonication, and centrifuged to remove cell debris. The supernatant was mixed with an equal volume of cold 95% ethanol, and the mixture was stirred for 20 min at 4 °C and then centrifuged. A mixture of cold 95% ethanol and $MgCl_2$ was slowly added to the resulting supernatant to give a final composition (assuming additive volumes) of 80% ethanol and 5 mM $MgCl_2$. The precipitate that formed overnight was recovered by centrifugation, resuspended in 10 mM potassium phosphate (pH 7.0), and centrifuged again to remove insoluble protein and nucleic acids. The resulting supernatant was loaded onto a DEAE-Sephacel column equilibrated with 1 mM Tris-phosphate (pH 7.0). The column was eluted with ∼500 mL of 10 mM Tris-phosphate (pH 7.0), followed by a 500 mL linear gradient from 10 to 200 mM Tris-phosphate (pH 7.0). Fractions containing enzyme were identified by SDS−PAGE and pooled. Solid ammonium sulfate was added to give 45% saturation (65% for D38H/D99A) (*35*), and the solution was left at 4 °C for 12−16 h. Precipitated protein was recovered by centrifugation, redissolved in a minimum volume of buffer, and dialyzed extensively against 10 mM potassium phosphate (pH 7.0). If necessary, the dialyzed protein was clarified by centrifugation and passage through a 0.2 μm filter.

Physical Characterization of Mutant Proteins. The purity of each protein was determined by discontinuous SDS−PAGE on 15% gels using the buffer system of Laemmli (*36*). The UV spectrum of each mutant was recorded in 10 mM potassium phosphate (pH 7.0), and the extinction coefficients at 280 nm were determined as described by Kuliopulos et al. (*12*).

Steady State Kinetics and pH−Rate Profiles. Aliquots of **1** in methanol (100 μL) were added to 3.00 mL of buffer previously equilibrated at 25.0 ± 0.2 °C in the spectrophotometer to give a final concentration of **1** of 5−120 μM. The reaction was initiated by addition of 5−25 μL of enzyme [prepared by diluting concentrated enzyme into 10 mM potassium phosphate buffer (pH 7.0) containing 0.25% BSA]. The enzyme concentration was such that 10% of the reaction occurred in ≤3 min. The reaction was monitored at 248 nm. Initial rates were determined from the first 5% or less of the reaction, and were corrected for nonenzymatic isomerization. The concentration of enzyme used in each experiment was generally calculated from the observed rate with 58 μM **1** in 34 mM potassium phosphate at pH 7.0, the specific activity for that mutant, and the subunit molecular weight (13 399 for WT, calculated from the gene sequence; *37*). Data, expressed as the initial rate per enzyme active site ($v_{init}/[E]_t$), were plotted in double-reciprocal form and fit to eq 1 with proportional weighting.

$$[E]_t/v_{init} = (1/k_{cat}) + (K_m/k_{cat})(1/[\mathbf{1}]) \qquad (1)$$

All buffers used to determine the kinetic constants were adjusted to constant ionic strength ($\mu = 0.1$ M) with KCl or NaCl. For D99N, the buffers were 34 mM acetate (pH 4.2−5.7), 34 mM MES (pH 6.0−6.6), 34 mM phosphate (pH 6.6−8.0), 5 mM CHES (pH 8.5−9.1), and 5 mM CAPS (pH 9.5−10.4). For D99A, the buffers were 34 mM acetate (pH 4.2−5.6), 34 mM phosphate (pH 6.0−8.5), 5 mM CHES (pH 9.0), 34 mM CHES (pH 9.3), and 5 mM CAPS (pH 10.2). For D38H/D99A, the buffers were 10 mM citrate (pH 3.5−4.5), 10 mM acetate (pH 4.5−6.0), 10 mM ADA or 10 mM phosphate (pH 6.0−7.5), 10 mM Tricine (pH 7.5−9.0),

10 mM CHES (pH 9.0−10.0), and 5 mM CAPS (pH 10.0−11.0). The stability of the D99A, D99N, and D38H/D99A mutants was verified at the extreme pH values by incubating the enzyme in the assay buffer at 25.0 ± 0.2 °C for 1 min and then initiating the reaction by addition of substrate or by incubating the enzyme in buffer for 1−2 min and assaying it at pH 7. The rate obtained was then compared to the rate obtained with substrate added before enzyme.

Native Gel Electrophoresis. Samples of WT and mutant KSI proteins were subjected to electrophoresis in 7.5% polyacrylamide gels at pH 6.00 ± 0.12 (34 mM sodium citrate), 6.78 ± 0.22 (34 mM sodium ADA), 7.54 ± 0.03 (34 mM sodium TES), 8.00 ± 0.09, and 8.54 ± 0.05 (34 mM Tris-HCl). Errors indicate differences in the pH from the beginning to the end of the run. In all cases, the anode was at the bottom of the gel. A peristaltic pump was used to recirculate the buffer between the upper and lower chambers. In addition, the lower buffer was mixed with a magnetic stirrer and chilled by placing the chamber in an ice bath. This arrangement was necessary to maintain the temperature of the upper buffer and gel at 18−20 °C. Each gel was prerun at 100 V for 1 h, after which samples (10 μL) containing 1.0−1.7 μg of protein, 10% glycerol, and 0.0033% bromophenol blue in 34 mM buffer were loaded onto the gel and run for 3−4 h at 100 V. The temperature and pH of upper and lower buffers were measured before loading the samples and again at the end of the run. A control gel was run at pH 8.5 without bromophenol blue to verify that it did not alter the mobility of any of the proteins. Gels were stained with Coomassie blue R-250 to reveal the protein bands and dried between sheets of cellophane. The mobility of D38N on each gel was determined by measuring the distance from the bottom of the protein band to the bottom of an adjacent D38N/D99A band and then dividing by the analogous distance between D38N/D99A and WT bands.

Enzyme Stability for Native Gel Experiments. Samples of each protein used in the native gel experiments (0.10−0.17 μg/μL) were incubated for 4 h at 18−21 °C in 30 mM sodium citrate (pH 6.0) and in 30 mM Tris-HCl (pH 8.5). Aliquots of WT, D99A, and D99N were removed at the beginning and end of the incubations, diluted into 10 mM potassium phosphate (pH 7.0) containing 2.5 mg/mL BSA, and assayed at pH 7.0 with 58 μM **1**, as described above. For D38N and D38N/D99A, the ability to bind equilenin (0.19 and 1.89 μM, respectively) was measured before and after the 4 h incubations.

RESULTS

Generation and Properties of the Mutant KSIs. The mutant genes (D99A, D99N, D38N/D99A, and D38H/D99A) were obtained by site-directed mutagenesis. Expression of the proteins in *E. coli* gave relatively high levels of the D99A and D38N/D99A mutants (>60 mg/L of culture) and somewhat lower amounts of the D99N and D38H/D99A proteins (∼10 mg/L of culture). All proteins were purified to >99% homogeneity, as determined by SDS−PAGE. Each mutant exhibits a UV spectrum in 10 mM potassium phosphate at pH 7.0 that is almost identical to that of native WT (*29*). Extinction coefficients at 280 nm for a 1 mg/mL solution at pH 7.0 are 0.355 cm^{-1} for D99N, 0.380 cm^{-1} for D99A, 0.413 cm^{-1} for D38N/D99A, 0.320 cm^{-1} for D38H/D99A, and 0.322 cm^{-1} for WT, similar to the published value for WT of 0.336 cm^{-1} (*38*). Specific activities for D99A (18 μmol min^{-1} mg^{-1}), D99N (2200 μmol min^{-1} mg^{-1}), and D38H/D99A (9.3 μmol min^{-1} mg^{-1}) were determined with 58 μM **1** in 34 mM potassium phosphate buffer (pH 7.0) and may be compared to a typical value of ∼50000 for WT (*29*). The D38N/D99A double mutant shows only barely detectable activity at an enzyme concentration of 4.6 μM, providing an upper limit for its specific activity of 2×10^{-4} μmol min^{-1} mg^{-1}. However, this mutant is able to bind ∼1 equiv per active site of the intermediate analogue equilenin, consistent with an intact active site.

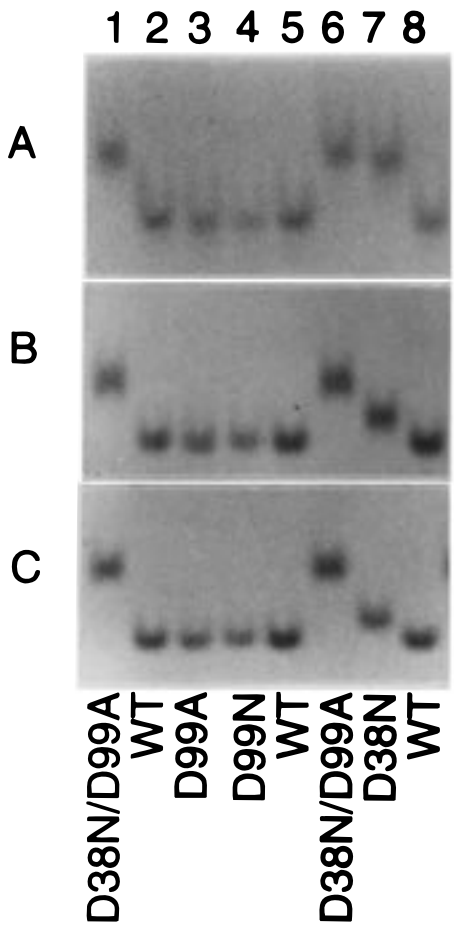


FIGURE 1: Native gel electrophoresis of WT and mutant KSI proteins at pH 6.8 (A), 8.0 (B), and 8.5 (C). The direction of migration was toward the anode at the bottom of the gels. On each gel, D38N/D99A is in lanes 1 and 6, WT is in lanes 2, 5, and 8, D99A is in lane 3, D99N is in lane 4, D38N is in lane 7.

Native Gel Electrophoresis. The relative charges of WT and mutants lacking Asp-99 and/or Asp-38 were determined by native gel electrophoresis according to the method of Langsetmo et al. (*39*). Mobilities of WT, D38N, D99A, D99N, and D38N/D99A were compared at several pH values ranging from 6.0 to 8.5. At all pH values examined, D99A and D99N have the same mobility as WT, whereas D38N/D99A migrates more slowly toward the anode (Figure 1). In contrast, D38N has the same mobility as D38N/D99A at both pH 6.0 (data not shown) and 6.8 (Figure 1A) but migrates more quickly at higher pHs. At pH 8.5, the mobility of D38N approaches that of WT (Figure 1C).

Table 1: Kinetic Constants for the Isomerization of 5-Androstene-3,17-dione (**1**) to 4-Androstene-3,17-dione (**3**) by KSI and Selected Mutant KSIs at 25 °C[a]

enzyme	limiting k_{cat} (s^{-1})	limiting k_{cat}/K_m (M^{-1} s^{-1})	pK_{E1}	pK_{E2}	pK_{ES1}	pK_{ES2}
WT[b]	$5.7 (\pm 0.2) \times 10^4$	$2.3 (\pm 0.2) \times 10^8$	4.57 ± 0.05[c]	~9.5[d]	4.74 ± 0.08[c]	~9.3[d]
D99N[e]	2130 ± 110	$1.00 (\pm 0.03) \times 10^7$	4.63 ± 0.05	−	5.37 ± 0.16	−
D99A[e]	18.7 ± 1.6	$1.0 (\pm 0.1) \times 10^5$	5.18 ± 0.09	−	5.45 ± 0.04	−
D38H[f]	564	1.3×10^7	4.37	8.5	3.41	7.52
D38H/D99A[e]	5.2 ± 0.5	$6.2 (\pm 0.4) \times 10^4$	4.72 ± 0.08	−	5.19 ± 0.10	−

[a] Reported errors are standard deviations. [b] Data from Pollack et al. (*24*), except as noted. [c] p*K* values for WT were determined with the slow, nonsticky substrate 5(10)-estrene-3,17-dione. [d] From data of Weintraub (*23*). [e] Conditions are described in Experimental Procedures. [f] Data from Holman and Benisek (*25*).

Experiments were carried out at pH 6.0 and 8.5 to verify that each mutant is stable under the conditions employed in the native gels. D99A, D99N, and WT all retain 100% of the activity after a 4 h incubation at pH 6.0. After 4 h at pH 8.5, D99N retains >80% of the activity while WT and D99A retain 95% of the activity. D38N and D38N/D99A show a <5% decrease in their abilities to bind equilenin after 4 h incubations at either pH 6.0 or 8.5.

Kinetic Constants and pH−Rate Profiles. Kinetic constants for the isomerization of **1** by each of the mutants were determined as a function of pH at 25.0 ± 0.2 °C. Over the pH range investigated, the enzymes are stable for the time required to make the rate measurements (<1 min). For all mutants lacking Asp-99, both k_{cat} and k_{cat}/K_m decrease at low pH but remain constant from pH 7.5 to >10 (Figure 2). It can be assumed that **1** is not a sticky substrate for these mutants, since the k_{cat}/K_m values are all at least 1 order of magnitude smaller than k_{cat}/K_m for WT, which reacts at nearly the diffusion-controlled rate. Thus, the plots of $(k_{cat}/K_m)_{obs}$ versus pH should provide the pK_as for the free enzymes (pK_E). The interpretation of the $(k_{cat})_{obs}$ versus pH plots is more complicated, but in the simplest case, they should give the pK_as for the enzyme−substrate complexes (pK_{ES}) (*40*−*42*). The observed kinetic parameters were fit to eqs 2 and 3 to give the kinetic constants and pK_a values, which are listed in Table 1 along with values determined previously for WT (*24*) and D38H (*25*).

$$(k_{cat}/K_m)_{obs} = (k_{cat}/K_m)/(1 + [H^+]/K_E) \quad (2)$$

$$(k_{cat})_{obs} = k_{cat}/(1 + [H^+]/K_{ES}) \quad (3)$$

DISCUSSION

pK_a of Asp-99. While the importance of the two catalytic residues Tyr-14 and Asp-38 of KSI has been appreciated for a long time (*12*−*14*), the recent elucidation of the structure of KSI revealed the presence of a third functional group, Asp-99, at the active site (*18*). This finding led us to postulate that the COOH of Asp-99 might donate a hydrogen bond to stabilize the reaction intermediate and transition state(s), as depicted in Scheme 1. However, since there is no diminution of activity for WT up to pH 9 (*23*, *24*), Asp-99 would need to have an anomalously high pK_a (>9). To test whether Asp-99 has an unusually high pK_a, we compared the electrophoretic mobilities of WT and several KSI mutants (D99A, D99N, D38N, and D38N/D99A) on nondenaturing gels at different pH values. In the absence of conformational differences between the mutants and WT, the mobility of each protein on a given gel depends upon only its charge (*39*).

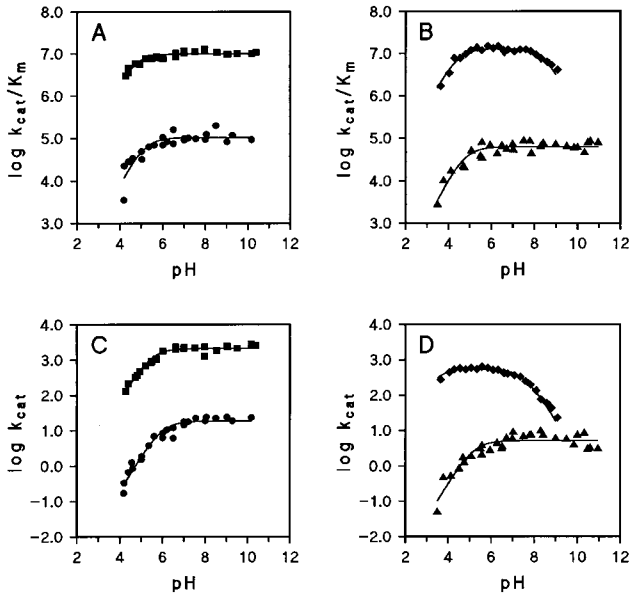


FIGURE 2: pH dependence of kinetic constants for the conversion of **1** to **3** by KSI mutants: (A) k_{cat}/K_m for D99A (●) and D99N (■), (B) k_{cat}/K_m for D38H/D99A (▲) and D38H [◆; data from Holman and Benisek (*25*)], (C) k_{cat} for D99A (●) and D99N (■), and (D) k_{cat} for D38H/D99A (▲) and D38H [◆; data from Holman and Benisek (*25*)]. The solid curves are nonlinear least-squares fits of the data to eq 2 or 3, with the parameters given in Table 1.

Several pieces of evidence suggest that there are no major structural changes in the mutants relative to WT. First, each of the mutant KSI proteins exhibits a UV spectrum at pH 7.0 that is almost identical to that of native WT. All spectra exhibit the distinct shoulder at 282−284 nm that is due to Tyr-14 in the hydrophobic environment of the active site (*43*); this feature is not present in the spectrum of denatured KSI. Second, the D99A, D99N, and D38H/D99A proteins are able to catalyze the isomerization of **1**, demonstrating that their active sites are intact. These catalytic activities are not simply due to contaminating WT because the activities of D99A and D99N (Figure 2A,C) differ markedly from that of WT at high pH (*23*). D38N/D99A, which has no significant catalytic activity, is able to bind the intermediate analogue equilenin, consistent with correct folding of this mutant. Finally, WT exists as a dimer at concentrations from 0.05 to 1 mg/mL (*38*, *44*, *45*), and the protein concentrations on our gels were within this range. If any of the mutants were present as monomers, they should migrate faster than WT, which was not observed.

In the interpretation of the results of the native gels, there are two hypothetical limiting cases that must be considered, bearing in mind that Asp-38 in WT has a pK_a of ~4.6 (*24*) and therefore will be ionized at all pH values examined. For

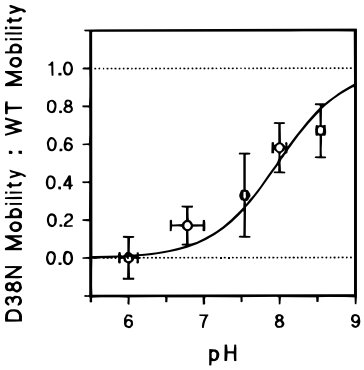


FIGURE 3: Relative mobility of D38N as a function of pH. Error bars represent the actual variation in pH from the beginning to the end of electrophoresis (on the *x*-axis) and the estimated error in measuring distances between protein bands (on the *y*-axis). Dotted lines indicate the relative mobilities of WT (upper dotted line) and D38N/D99A (lower dotted line). The solid curve is a nonlinear least-squares fit of the data to eq 4, with a pk_a of 7.9.

the first scenario, assume that Asp-99 is ionized in both WT and D38N. WT would thus have two more negative charges per subunit than the double mutant D38N/D99A and would migrate more quickly toward the bottom of the gel. The single mutants D99A, D99N, and D38N would have only one more negative charge per subunit than D38N/D99A and would move halfway between it and WT. For the second case, assume that Asp-99 is protonated in both WT and D38N. The charge difference per subunit between D38N/D99A and WT would now be −1. The single mutants D99A and D99N would have the same charge, and thus the same mobility as WT, while D38N would have the same charge and mobility as the double mutant D38N/D99A.

The native gel run at pH 6.8 (Figure 1A) shows conclusively that D99A and D99N have the same mobility as WT and that D38N migrates with D38N/D99A, as predicted by the second scenario. Therefore, Asp-99 in both WT and D38N is protonated at pH 6.8.[4] Furthermore, WT has the same mobility as the single mutants D99A and D99N at all pH values examined. Consequently, the pK_a of Asp-99 in WT must be significantly greater than 8.5, the highest pH examined. In contrast, the mobility of D38N increases with increasing pH until it migrates almost as rapidly as WT at pH 8.5, suggesting that some amino acid in this mutant is being titrated. Because the D38N/D99A mutant does not exhibit this behavior, the most likely candidate is Asp-99. The mobility of D38N as a function of pH (Figure 3) can be fit to a titration curve (eq 4) with a pK_a of ∼8.

$$\text{D38N mobility/WT mobility} = 10^{(\text{pH}-\text{p}K)}/[1 + 10^{(\text{pH}-\text{p}K)}] \quad (4)$$

The high pK_a of Asp-99 in both D38N and WT can be accounted for by the extremely hydrophobic environment in which this amino acid side chain finds itself (*18*). In addition, the electrostatic effect of negatively charged Asp-38 in proximity (∼4 Å) to Asp-99 would be expected to increase the pK_a of the latter in WT.

Highly perturbed pK_a values of amino acid side chain carboxylates have been observed in other enzymes. *E. coli* thioredoxin Asp-26, which is completely buried in a hydrophobic groove, has a pK_a of ∼7.5 in the oxidized form of the protein (*39*), and in the reduced form, it has microscopic pK_as that depend on the ionization state of the interacting residue Cys-32 (*46*). If Cys-32 is un-ionized, Asp-26 shows a pK_a of 7.5, but in the presence of anionic Cys-32, the pK_a of Asp-26 is 9.2. A pK_a of 9.2 has been reported for the active site residue Glu-139 in enoyl-CoA hydratase from *E. coli* (*47*); the corresponding residue in the rat liver enzyme, Glu-164, has a pK_a of 8.45 in an enzyme−inhibitor complex (*48*). A pK_a of ∼8 has also been observed for the Asp-25 and -25′ residues in an HIV protease−inhibitor complex, in which these residues are completely buried (*49*). In addition, the Asp residue in a synthetic protein-based polymer was shown to have a pK_a of 10 in the hydrophobic environment created by five proximal Phe residues (*50*).

The finding that Asp-99 is protonated in WT under normal assay conditions strongly supports a hydrogen bonding role for the carboxyl group of this amino acid. The recent 2.5 Å X-ray structure determination of the D38N mutant of KSI from *P. putida* with the intermediate analogue equilenin bound at the active site (*21*) is consistent with direct hydrogen bonding of Asp-99 to the dienolate oxygen (Scheme 1). In this complex, both the OH of Tyr-14 and the COOH of Asp-99 are within hydrogen bonding distance of O-3 of equilenin. The high pK_a of Asp-99 could also explain the observation of Li et al. (*51*), who detected pK_as of 9.5 and 11.5 by fluorescence quenching of the Y55F/Y88F mutant of KSI. Because the latter pK_a was also observed by UV absorbance at 295 nm, it was assigned to Tyr-14 (the only tyrosine in this protein); however, the pK_a of 9.5 remained an enigma. Our results strongly indicate that this pK_a is due to Asp-99.

pH−Rate Profiles. The high pK_a of Asp-99 provides an attractive explanation for the decrease in k_{cat}/K_m at high pH (pK_{E2} = 8.5) for D38H (*25*) and the loss in activity and/or denaturation of WT at pH >9 (*23*). These kinetic results had been puzzling for a long time since the only known candidate for this ionization, Tyr-14, had been ruled out for both WT (*51*) and the D38H mutant (*25*). To test the hypothesis that these decreases in activity at high pH are associated with ionization of Asp-99, we determined the pH−rate profiles for the D99A, D99N, and D38H/D99A KSI mutants. The D99A mutation replaces the side chain of Asp with Ala, which lacks the carboxylic acid group proposed to interact with the dienolate intermediate. The amide of Asn introduced by the D99N mutation preserves the side chain volume of Asp as well as the hydrogen bonding potential with the dienolate intermediate, and thus mimics a protonated aspartic acid. Because the D38H mutant gives a lower value of pK_{E2} than WT, it is possible to see changes in kinetic constants at high pH without the complication of denaturation seen with WT. Thus, a comparison of the pH−rate profiles for D38H with D38H/D99A allows a direct determination of whether pK_{E2} is associated with Asp-99.

For each of the mutants that lacks Asp-99 (D99A, D99N, and D38H/D99A), both k_{cat} and k_{cat}/K_m depend on only one titratable group in the pH range examined (Figure 2). In contrast, the pH−rate profiles for D38H exhibit decreases

[4] An alternate explanation of this result would be that Asp-99 is ionized in WT and mutation to Ala or Asn causes deprotonation of another residue, thus maintaining the same net charge. It is unlikely that this has occurred since the only ionizable groups in the neighborhood of Asp-99 are Tyr-14 and Tyr-55, and UV spectra of D99A and D99N show no absorbance at 293 nm from a tyrosinate ion.

in k_{cat} and k_{cat}/K_m at high pH, while WT, with an ionizable Asp-99, is unstable at pH >9 and loses activity (*24*). These results clearly demonstrate that the decrease in the kinetic constants for D38H at high pH is due to ionization of Asp-99. The inactivation of WT at high pH is likely due to the introduction of a negative charge upon ionization of Asp-99 within a few angstroms of the negative charge on Asp-38 ($pK_a = 4.6$).

Hydrogen Bonding by Asp-99. The mechanism of Scheme 1 predicts that replacement of Asp-99 by another amino acid capable of hydrogen bonding should have a less deleterious effect on catalytic activity than replacement by Ala. The kinetic constants presented in Table 1 show that the D99N mutant is intermediate in activity between WT and D99A. The limiting values at high pH of both k_{cat} and k_{cat}/K_m for D99N are ~25-fold lower than those for WT, in accord with the expected ability of an amide group to hydrogen bond more weakly than a carboxylic acid. The greater decrease in limiting values of k_{cat} and k_{cat}/K_m for D99A relative to WT (~2600-fold) corresponds to ~4.6 kcal/mol of stabilization energy, and is consistent with a hydrogen bonding role for Asp-99. These results can be compared with the recently reported decreases in k_{cat} and k_{cat}/K_m for the D99A and D99N mutants of KSI from *P. putida* relative to those of WT of ~100- and ~3-fold, respectively (*52*).

Alternative Mechanisms. The results presented here, coupled with the structural information (*18*, *21*), lead to the conclusion that protonated Asp-99 is an important residue for the isomerization of Δ^5-3-oxosteroids catalyzed by KSI. On the basis of computer docking of a substrate molecule into the active site of the solution structure of KSI, we found that Asp-99 is located ~3 Å from the carbonyl oxygen (*18*), leading to the postulate that Asp-99 stabilizes the intermediate dienolate ion (**2**) by direct hydrogen bonding. The X-ray structure of the *P. putida* KSI−equilenin complex (*21*) is also consistent with this mechanism. However, Zhao et al. (*22*) have proposed an alternate mechanism that involves Asp-99 hydrogen bonding to Tyr-14, which in turn hydrogen bonds to O-3 of the steroid. We believe that the argument of Zhao et al. (*22*) supporting their proposed "catalytic diad" mechanism is not a strong one. The four pieces of evidence they present are given below, along with our comments.

(1) "H-bond donation by Tyr-14 is compensated by another H-bond donor as detected by UV resonance Raman and UV absorption spectra of Tyr-14 with and without steroid binding." Although there may be a hydrogen bond to Tyr-14 from another amino acid at the active site, there is no evidence that it is Asp-99. In fact, in the *P. putida* enzyme, there is a hydrogen bond to Tyr-14, but it is donated by Tyr-55. The solution structure of KSI from *P. testosteroni* also reveals that the oxygens of Tyr-14 and Tyr-55 are within H-bonding distance (*18*). Interestingly, replacement of Tyr-55 by Phe (Y55F) results in only a 4-fold reduction in k_{cat} (*12*), suggesting that such a hydrogen bond is only minimally important for catalytic activity. Possibly, the loss of the hydrogen bond between Tyr-55 and Tyr-14 in this mutant is compensated by a hydrogen bond between an active site water molecule and Tyr-14.

(2) "The side chain of Asp-99 is close to the aromatic ring of Tyr-14 as indicated by NOEs from the Cβ protons of Asp-99 to Tyr-14 Hϵ, and by the half quenching of the fluorescence of Tyr-14 on titrating a group in the free enzyme with a pK_a of 9.5, presumably Asp-99." These workers report distances of 2.8 ± 0.2 Å between Hϵ of Tyr-14 and the Hβ protons of Asp-99, based on the relative intensities of NOE cross-peaks in a three-dimensional ^{13}C-edited NOESY−HSQC spectrum. However, such narrow distance limits are unrealistic, given the fact that the intensities are influenced by numerous factors, including spin diffusion and differences in t_2 relaxation rates (*53*). Most laboratories employ much wider distance bounds of ±1.6 Å when attempting to model weak NOEs (*54*). Furthermore, we have been unable to detect these NOE cross-peaks in the NOESY HMQC spectrum of uncomplexed KSI (Z. R. Wu, M. F. Summers, and R. M. Pollack, unpublished results).

(3) "The γ-COOH proton of Asp-99 is near the ζ-OH proton of Tyr-14 based on NOEs." Cross-peaks in the two-dimensional NOESY data involving rapidly exchanging OH protons are difficult to interpret properly due to the likelihood of rapid OH−OH proton exchange (relative to the mixing period employed), which would give rise to artifactual NOEs. Thus, this conclusion is only tentative.

(4) "The H-bond donated by Asp-99 is much stronger than that donated by Tyr-14 as indicated by the greater deshielding (6.2 versus 1.8 ppm) and lower fractionation factor (0.34 versus 0.97) of H_a in comparison to H_c. These two protons are linked in an H-bonded network since both of their resonances shift upfield on lowering the pK_a of the intermediate analogue by 1.6 units." These results are also consistent with the model of Scheme 1, in which both Asp-99 and Tyr-14 H-bond *directly* to O-3 of the steroid, and thus cannot be used to differentiate between the mechanisms. Moreover, the assignments of H_a to Asp-99 and H_c to Tyr-14 appear to be based on insufficient evidence.

CONCLUSION

Several lines of evidence demonstrate that Asp-99 of KSI has a high pK_a and that the protonated form of this group is involved in the catalytic mechanism of KSI. We have shown that (1) Asp-99 in WT has a pK_a significantly higher than 8.5, (2) the mutants lacking this group (D99A, D99N, and D38H/D99A) show no diminution of rate at high pH, and (3) the D99N mutant is significantly more active than the D99A mutant. These results provide strong support for electrophilic assistance by Asp-99 in stabilizing the transition state(s) of the KSI-catalyzed reaction. In conjunction with the NMR structure of KSI from *P. testosteroni* (*18*) and the X-ray structure of the homologous KSI from *P. putida* (*21*), these findings are consistent with stabilization of the intermediate dienolate ion and the transition state(s) by direct hydrogen bonding from both Tyr-14 and Asp-99. The decrease in activity (~45000-fold in k_{cat} and ~17000-fold in k_{cat}/K_m) for the Y14F mutant compared to WT (*12*) suggests that the contribution to stability from Tyr-14 is 5.7−6.3 kcal/mol, somewhat greater than that from Asp-99 (~4.6 kcal/mol). Thus, two hydrogen bonds to O-3 of the dienolate intermediate with energy contributions of 4−6 kcal/mol, rather than a very strong hydrogen bond from a single amino acid, can account for the ~10 kcal/mol of stabilization energy supplied by KSI. Evidence that has been cited as supporting the alternative mechanism, with a chain of hydrogen bonds from Asp-99 to Tyr-14 to O-3, is also consistent with Scheme 1.

ACKNOWLEDGMENT

We thank Dr. Grzegorz Blotny, Dr. Ioanna P. Petrounia, and Dr. Lin Qi for gifts of 5-androstene-3,17-dione, D38N, and WT, respectively. We also thank Professor M. F. Summers for helpful discussions.

NOTE ADDED IN PROOF

Cho et al. (*55*) have recently published the crystal structure of KSI from *P. testosteroni*. Their structure is consistent with the one that we determined by NMR methods, and supports the mechanism of Scheme 1. The alternative mechanism of Scheme 2 may be ruled out by the distance between the oxygens of Asp-99 and of Tyr-14 (3.87 Å), which is too far for hydrogen bond formation between these residues.

REFERENCES

1. Kresge, A. J. (1975) *Acc. Chem. Res*. *8*, 354.
2. Gerlt, J. A., and Gassman, P. G. (1993) *J. Am. Chem. Soc. 115*, 11552.
3. Gerlt, J. A., and Gassman, P. G. (1993) *Biochemistry 32*, 11943.
4. Cleland, W. W., and Kreevoy, M. M. (1994) *Science 264*, 1887.
5. Gerlt, J. A., Kreevoy, M. M., Cleland, W. W., and Frey, P. A. (1997) *Chem. Biol. 4*, 259.
6. Guthrie, J. P., and Kluger, R. (1993) *J. Am. Chem. Soc. 115*, 11569.
7. Warshel, A., Papazyan, A., and Kollman, P. A. (1995) *Science 269*, 102.
8. Shan, S., Loh, S., and Herschlag, D. (1996) *Science 272*, 97.
9. Guthrie, J. P. (1996) *Chem. Biol. 3*, 163.
10. Shan, S., and Herschlag, D. (1996) *J. Am. Chem. Soc. 118*, 5515.
11. Hawkinson, D. C., Eames, T. C. M., and Pollack, R. M. (1991) *Biochemistry 30*, 10849.
12. Kuliopulos, A., Mildvan, A. S., Shortle, D., and Talalay, P. (1989) *Biochemistry 28*, 149.
13. Benisek, W. F., Ogez, J. R., and Smith, S. B. (1980) *Ann. N.Y. Acad. Sci. 346*, 115.
14. Bounds, P. L., and Pollack, R. M. (1987) *Biochemistry 26*, 2263.
15. Zawrotny, M. E., Hawkinson, D. C., Blotny, G., and Pollack, R. M. (1996) *Biochemistry 35*, 6438.
16. Viger, A., Coustal, S., and Marquet, A. (1981) *J. Am. Chem. Soc. 103*, 451.
17. Hawkinson, D. C., Pollack, R. M., and Ambulos N. P. (1994) *Biochemistry 33*, 12172.
18. Wu, Z. R., Ebrahimian, S., Zawrotny, M. E., Thornburg, L. D., Perez-Alvarado, G. C., Brothers, P., Pollack, R. M., and Summers, M. F. (1997) *Science 276*, 415.
19. Linden, K. G., and Benisek, W. F. (1986) *J. Biol. Chem. 261*, 6454.
20. Molnar, I., Choi, K. P., Yamashita, M., and Murooka, Y. (1995) *Mol. Microbiol. 15*, 895.
21. Kim, S. W., Cha, S.-S., Cho, H.-S., Kim, J.-S., Ha, N.-C., Cho, M.-J., Joo, S., Kim, K. K., Choi, K. Y., and Oh, B.-H. (1997) *Biochemistry 36*, 14030.
22. Zhao, Q., Abeygunawardana, C., Gittis, A. G., and Mildvan, A. S. (1997) *Biochemistry 36*, 14616.
23. Weintraub, H., Alfsen, A., and Baulieu, E.-E. (1970) *Eur. J. Biochem. 12*, 217.
24. Pollack, R. M., Bantia, S., Bounds, P. L., and Koffman, B. D. (1986) *Biochemistry 25*, 1905.
25. Holman, C. M., and Benisek, W. F. (1995) *Biochemistry 34*, 14245.
26. Eames, T. C. M., Pollack, R. M., and Steiner, R. F. (1989) *Biochemistry 28*, 6269.
27. Pollack, R. M., Zeng, B., Mack, J. P. G., and Eldin, S. (1989) *J. Am. Chem. Soc. 111*, 6419.
28. Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual*, 2nd ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
29. Kawahara, F. S., Wang, S.-F., and Talalay, P. (1962) *J. Biol. Chem. 237*, 1500.
30. Taylor, J. W., Ott, J., and Eckstein, F. (1985) *Nucleic Acids Res. 13*, 8764.
31. Nakamaye, K., and Eckstein, F. (1986) *Nucleic Acids Res. 14*, 9679.
32. Sayers, J. R., Krekel, C., and Eckstein, F. (1992) *BioTechniques 13*, 592.
33. Zawrotny, M. E., Ambulos, N. P., Lovett, P. S., and Pollack, R. M. (1991) *J. Am. Chem. Soc. 113*, 5890.
34. Brooks, B., and Benisek, W. F. (1994) *Biochemistry 33*, 2682.
35. Englard, S., and Seifter, S. (1990) *Methods Enzymol. 182*, 285.
36. Laemmli, U. K. (1970) *Nature 227*, 680.
37. Choi, K. Y., and Benisek, W. F. (1988) *Gene 69*, 121.
38. Benson, A. M., Suruda, A. J., and Talalay, P. (1975) *J. Biol. Chem. 250*, 276.
39. Langsetmo, K., Fuchs, J. A., and Woodward, C. (1991) *Biochemistry 30*, 7603.
40. Knowles, J. R. (1976) *Crit. Rev. Biochem. 4*, 165.
41. Cleland, W. W. (1977) *Adv. Enzymol. Relat. Areas Mol. Biol. 45*, 273.
42. Fersht, A. (1984) *Enzyme Structure and Mechanism*, 2nd ed., W. H. Freeman, New York.
43. Zhao, Q., Li, Y.-K., Mildvan, A. S., and Talalay, P. (1995) *Biochemistry 34*, 6562.
44. Weintraub, H., Vincent, F., and Baulieu, E. E. (1973) *FEBS Lett. 37*, 82.
45. Tivol, W. F., Beckman, E. D., and Benisek, W. F. (1975) *J. Biol. Chem. 250*, 271.
46. Chivers, P. T., Prehoda, K. E., Volkman, B. F., Kim, B.-M., Markley, J. L., and Raines, R. T. (1997) *Biochemistry 36*, 14985.
47. He, X.-Y., and Yang, S.-Y. (1997) *Biochemistry 36*, 11044.
48. D'Ordine, R. L., Bahnson, B. J., Tonge, P. J., and Anderson, V. E. (1994) *Biochemistry 33*, 14733.
49. Yamazaki, T., Nicholson, L. K., Torchia, D. A., Wingfield, P., Stahl, S. J., Kaufman, J. D., Eyermann, C. J., Hodge, C. N., Lam, P. Y. S., Ru, Y., Jadhav, P. K., Chang, C.-H., and Weber, P. C. (1994) *J. Am. Chem. Soc. 116*, 10791.
50. Urry, D. W., Gowda, D. C., Peng, S., Parker, T. m., Jing, N., and Harris, R. D. (1994) *Biopolymers 34*, 889.
51. Li, Y.-K., Kuliopulos, A., Mildvan, A. S., and Talalay, P. (1993) *Biochemistry 32*, 1816.
52. Kim, S. W., Joo, S., Choi, G., Cho, H.-S., Oh, B.-H., and Choi, K. Y. (1997) *J. Bacteriol. 179*, 7742.
53. Neuhaus, D., and Williamson, M. (1989) *The Nuclear Overhauser Effect In Structural and Conformational Analysis*, VCH, New York.
54. Wüthrich, K. (1986) *NMR of Proteins and Nucleic Acids*, Wiley, New York.
55. Cho, H.-S., Choi, G., Choi, K. Y., and Oh, B.-H. (1998) *Biochemistry 37*, 8325−8330.

BI980099A