Importance of Lysine-286 at the NADP Site of Glutamate Dehydrogenase from *Salmonella typhimurium*†

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Received March 11, 1992; Revised Manuscript Received June 3, 1992

ABSTRACT: Affinity labeling studies of NADP$^+$-glutamate dehydrogenase from *Salmonella typhimurium* have shown that the peptide Leu-282–Lys-286 is located near the coenzyme site [Haeffner-Gormley et al. (1991) *J. Biol. Chem. 266*, 5388–5394]. The present study was undertaken to evaluate the role of lysine-286. The mutant enzymes K286R, K286Q, and K286E were prepared by site-directed mutagenesis, expressed in *Escherichia coli*, and purified. The V_{max} values (micromoles of NADPH per minute per milligram of protein) were similar for WT (270), K286R (529), K296Q (409), and K286E (382) enzymes. As measured at pH 7.9, the K_m value for NADPH was much greater for K286E (280 μM) than for WT (9.8 μM), K286R (30 μM), or K286Q (66 μM) enzymes. The efficiencies (k_{cat}/K_m) of the WT and K286R mutant were similar (1.2×10^3 min^{-1} μM^{-1} and 1.0×10^3 min^{-1} μM^{-1}, respectively) while those of K286Q (0.30×10^3 min^{-1} μM^{-1}) and K286E (0.07×10^3 min^{-1} μM^{-1}) were greatly reduced. The decreased efficiency of the K286E mutant results from the increase in $K_{m\text{-NADPH}}$, consistent with a role for a basic residue at position 286 which enhances the binding of NADPH. Plots of V_{max} vs pH showed the pH optima to be 8.1–8.3 for all enzymes at saturating NADPH concentrations. A 40-fold increase in $K_{m\text{-NADPH}}$ for K286E was observed as the pH increased from 5.98 to 8.08, from which a unique pK_e of 6.5 was calculated. The wild-type enzyme can use NADH as the coenzyme, but k_{cat} is 10-fold lower and K_m is 20-fold higher than for NADPH as the coenzyme. In contrast to the major differences in $K_{m\text{-NADPH}}$, wild-type and mutant enzymes have similar K_m values for NADH. Thus, changing the amino acid at position 286 influences the interaction of the enzyme with the 2′-phosphate of the coenzyme. A possible role for lysine-278 in the binding of coenzyme was also investigated using the mutant K278Q. The kinetic parameters of this mutant enzyme were all similar to those of wild-type glutamate dehydrogenase indicating that, in contrast to lysine-286, lysine-278 is not associated with coenzyme binding.

Glutamate dehydrogenase (EC 1.4.1.4) from *Salmonella typhimurium* is an NADP$^+$-dependent enzyme. The sequence of the enzyme (Bansal et al., 1989) exhibits considerable similarity, indicative of homology, with other NADP$^+$-glutamate dehydrogenases, especially the enzyme from *Escherichia coli* (Valle et al., 1984; Blumenthal et al., 1975; Wootton et al., 1975). The gene encoding glutamate dehydrogenase from *S. typhimurium* has been cloned (Miller & Brenchley, 1984), its nucleotide sequence has been determined (Bansal et al., 1989) and wild-type as well as mutant enzymes have been expressed in *E. coli* and purified (Haeffner-Gormley et al., 1991). No three-dimensional structure of NADP$^+$-glutamate dehydrogenase from any source has been reported. However, the *E. coli* enzyme has been predicted to have the $\beta\alpha\beta$ helical motif (Kuroda et al., 1990) characteristic of nucleotide coenzyme binding sites (Wierenga et al., 1985), and therefore, the enzyme from *S. typhimurium* might also be expected to have this motif.

Recent studies have established a role for basic amino acids in the binding of the coenzyme NADP(H) in various enzymes. These basic residues interact with the 2′-phosphate group of the coenzyme. This evidence has been obtained through studies employing X-ray crystallography (Filman et al., 1982; Pai et al., 1988; Hurley et al., 1991), NMR (Feeney et al., 1975; Mas & Colman, 1984), electron density (Bajorath et al., 1991), and site-directed mutagenesis (Adams et al., 1989; Huang et al., 1990).

Previous studies in our laboratory have shown that NADP$^+$-glutamate dehydrogenase from *S. typhimurium* can be partially inactivated by the nucleotide affinity labels 2-[(4-bromo-2,3-dioxobutyl)thio]-1,N^6-ethenoadenosine 2′,5′-bisphosphate (Haeffner-Gormley et al., 1991) and 2-[(4-bromo-2,3-dioxobutyl)thio]adenosine 2′,5′-bisphosphate (Haeffner-Gormley et al., 1992a). By using mutant enzymes it was shown that the modification of cysteine-283 was responsible for the enzyme's inactivation, although cysteine-283 itself is not essential for activity. The presence of NADPH prevented modification and inactivation, indicating that cysteine-283 is near the coenzyme binding site. The isolated modified tryptic peptide was Leu^{282}–Lys^{286}. Since lysine-286 is probably also in the region of the coenzyme binding site of glutamate dehydrogenase from *S. typhimurium*, the present investigation was undertaken to evaluate a possible role for this basic amino acid residue in coenzyme binding. Three mutant enzymes were prepared by site-directed mutagenesis for this purpose. Lysing-286 was substituted by arginine (K286R), glutamine (K286Q), and glutamic acid (K286E) in order to assess the effect of the amino acid charge on coenzyme binding, catalysis, and coenzyme specificity. An additional mutant enzyme, K278Q, was also engineered and examined to evaluate whether lysine-278 has a role in the binding of NADPH. A preliminary

† This work was supported in part by U.S.P.H.S. Grants DK37000 (R.F.C.) and GM 24658 (H.Z.).

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0006-2960/92/0431-7807$03.00/0

version of this work has been presented (Haeffner-Gormley et al., 1992b).

EXPERIMENTAL PROCEDURES

Materials. β-Nicotinamide adenine dinucleotide phosphate, reduced form (NADPH), and α-ketoglutaric acid were obtained from Sigma. Ultrapure-grade Tris buffer was from Schwarz/Mann Biotech. Matrex Red gel was from Amicon Corp. The HPLC TSK 4000 SW column was made by Toya Soda. HPLC-grade acetonitrile was from Mallinckrodt. HPLC buffer was prepared from water purified on a Milli-Q system and was filtered using an HA filter (Millipore). The electrophoresis protein calibration kit was from Pharmacia LKB.

Site-Directed Mutagenesis and Preparation of Extracts. The gene encoding *S.typhimurium* glutamate dehydrogenase was cloned and expressed in *E. coli* (Bansal et al., 1989). Site-directed mutagenesis was performed by the method of Kunkel et al. (1987) using previously described plasmids and phagemids (Haeffner-Gormley et al., 1991). Mutagenic 19-mer oligonucleotides were synthesized to give the K286E and K286Q mutant glutamate dehydrogenases; the respective amino acid codons for position 286 were GAA and CAA, replacing the original lysine AAA codon. The mutant K286R was prepared using a 30-mer oligonucleotide with an amino acid codon of CGC. An additional mutant, K278Q, was produced using a 19-mer oligonucleotide with the final amino acid codon CAG replacing the original lysine-278 codon of AAG. The mutations were verified by nucleotide sequence analyses of the gene between codons 275 and 321 (Sanger et al., 1977). Plasmid-bearing *E. coli* DH5α cells were grown and extracts prepared as previously described (Haeffner-Gormley et al., 1991).

Purification of Wild-Type and Mutant Enzymes. Wild-type and the mutant enzymes K286R, K286Q, and K283E are purified from the crude extracts in two steps as reported previously for wild-type and other glutamate dehydrogenase mutant enzymes (Bansal et al., 1989; Haeffner-Gormley et al., 1991). The extracts (1.35–1.75 g of protein) were diluted 2-fold with 0.1 M potassium phosphate buffer, pH 7.0, and applied to a Matrex Red column (2.5 × 40 cm) equilibrated with the same buffer. After removal of unwanted protein with this buffer, active enzyme was eluted with 1.0 M KCl in the same buffer. The fractions of highest specific activity were dialyzed against 0.1 M potassium phosphate, pH 7.0. The samples were concentrated in Centricon-10 microconcentrators (Amicon). Samples containing 8–10 mg of protein/mL were further purified by gel filtration on a TSK 4000 SW HPLC column (0.75 × 50 cm), eluted with the same phosphate buffer, pH 7.0, at a flow rate of 0.5 mL/min. A Varian Model 5000 HPLC system was used and 1-min fractions were collected. The fractions of highest specific activity were used in the studies reported.

Polyacrylamide Gel Electrophoresis. The purity of the wild-type and mutant glutamate dehydrogenases was evaluated on polyacrylamide gels. A discontinuous gel system containing 0.1% sodium dodecyl sulfate (SDS)[1] was used (Blackshear, 1984). For this system a stock gel solution was prepared containing 37.5% acrylamide and 0.5% bisacrylamide. A 5.4% acrylamide stacking gel was prepared in 0.12 M Tris-HCl buffer, pH 6.8. The separating gel was 15% acrylamide in 0.38 M Tris-HCl, pH 8.8. The electrode buffer, in a total volume of 1 L, contained 6 g of Tris, 28.8 g of glycine, and 5 mL of 20% SDS and had a final pH of 8.3. Electrophoresis was performed on a Mini-protean II (Bio-Rad) unit in a 4 °C cold room using a constant voltage (100 V). Gels were stained with 0.1% Coomassie blue in 50% methanol. The proteins used as molecular weight standards were phosphorylase *b* (M_r 94 000), bovine serum albumin (M_r 67 000), ovalbumin (M_r 43 000), carbonic anhydrase (M_r 30 000), soybean trypsin inhibitor (M_r 20 100), and α-lactalbumin (M_r 14 400).

Protein Analyses. Glutamate dehydrogenase protein concentrations were determined using $E^{0.1\%}_{280nm} = 1.17$. N-Terminal amino acid analyses were obtained on an automated gas-phase protein sequencer (Applied Biosystems, Model 470A) equipped with an on-line phenylthiohydantoin analyzer, Model 120A.

Enzyme Assays and Kinetic Studies. The standard assay system contained 50 mM potassium phosphate (pH 8.0), 5 mM α-ketoglutarate (adjusted to pH 7.0), 50 mM ammonium chloride (adjusted to pH 7.9), and 100 μM NADPH in a final volume of 1.0 mL; this assay solution had a final pH of 7.87. The activity at 25 °C was determined by measuring the initial rate of oxidation of NADPH at 340 nm ($\epsilon = 6.22 \times 10^3$ M^{-1} cm^{-1}) using a Gilford Model 240 spectrophotometer.

To determine the K_m values for the several substrates, the concentrations of two substrates were held constant while the third was varied; the details are given in Results. At higher concentrations of NADPH it was necessary to use a wavelength of either 375 nm ($\epsilon = 1.97 \times 10^3$ M^{-1} cm^{-1}) or 390 nm ($\epsilon = 0.46 \times 10^3$ M^{-1} cm^{-1}). In all cases, there was a linear dependence of absorbance on NADPH concentration under the conditions used. For the studies using 2000 μM NADPH, rates were always measured at 390 nm. The observed velocities were usually expressed as micromoles of NADPH per minute per milligram of protein. Lineweaver–Burk plots were used to obtain K_m and apparent V_{max}. To calculate k_{cat} from V_{max}, a subunit molecular weight of 45 000 was used.

Direct Comparison of Activities with NADPH and NADH as Coenzymes. Wild-type, K286R, K286Q, and K286E glutamate dehydrogenases were assayed in the same experiment in 50 mM potassium phosphate (pH 8.0) containing 50 mM NH_4Cl, 2 mM α-ketoglutarate, and 2000 μM of either NADPH and NADH. The enzyme preparations were diluted to yield the same protein concentration, 0.5 and 4.0 μg of enzyme/mL of assay solution for the NADPH and NADH reactions, respectively. Multiple rate determinations were averaged. The change in absorbance at 390 nm was measured and the observed velocities [micromoles NAD(P)H per minute per micromole of enzyme subunit] were extrapolated to V_{max} using the relationship $V_{max} = V[1 + (K_{m\text{-}NAD(P)H})/2000\ \mu M]$.

pH Profile Studies. A series of potassium phosphate buffers of pH 5.5–8.0, as well as a series of Tris-HCl buffers of pH 8.0–9.0, were prepared. The ionic strength was maintained constant at 0.2 M in the assay by the addition of KCl to the phosphate buffers and of potassium phosphate (pH 8.0) to the Tris-HCl buffers. The final concentrations in the assay solutions were 50 mM buffer, 50 mM NH_4Cl and 5 mM α-ketoglutarate; the concentration of NADPH was varied for wild-type and mutant glutamate dehydrogenases as explained in Results. The final pH was determined after measurement of the reaction rate.

The $K_{m\text{-}NADPH}$ for wild-type, K286Q, and K286E enzymes was also determined at selected pH values. The buffers used were those from the pH profile experiments and the ionic strength was 0.2 M.

[1] Abbreviations: SDS, sodium dodecyl sulfate; $K_{m\text{-}\alpha KG}$, Michaelis constant for α-ketoglutarate; PAGE, polyacrylamide gel electrophoresis; DHFR, dihydrofolate reductase.

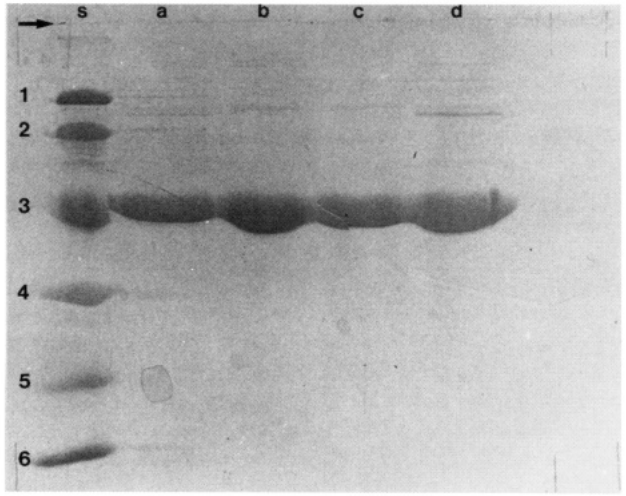

FIGURE 1: Gel electrophoresis of wild-type and mutant glutamate dehydrogenases. Gels were run in a discontinuous 0.1% SDS system. The stacking gel was 5.4% acrylamide, pH 6.8, and the separating gel was 15% acrylamide, pH 8.8. The glutamate dehydrogenase samples contained 8–9 μg of protein. The origin is at the top of the gel. (a) K286Q, (b) K286E, (c) K286R, and (d) wild-type enzyme. The standard proteins (s) had the following molecular weights: (1) 94 000, (2) 67 000; (3) 43 000, (4) 30 000, (5) 20 100, and (6) 14 400.

RESULTS

Purification of Mutant Glutamate Dehydrogenases. The mutant enzymes K286R, K286Q, and K286E were all bound by the Matrex Red column which was equilibrated with 0.1 M potassium phosphate buffer at pH 7. All the mutants, eluted by the addition of 1 M KCl to the same buffer, had elution profiles (absorbance 280 nm) similar to that of wild-type enzyme (data not shown). Since the Matrex Red gel often functions as an affinity column for NADP-dependent enzymes (Watsun et al., 1978), this observation suggested that the coenzyme binding site was not completely disrupted in these mutants, although other factors also contribute to protein binding on dye-ligand columns. Since elution was accomplished by an abrupt change in salt concentration, rather than by a gradient, slight differences among the mutant enzymes in the strength of binding to the column would not have been observed. Elution from the HPLC gel-filtration column also produced similar chromatographs for the mutant and wild-type enzymes, with the highest specific activity being found in corresponding fractions. Aliquots from these fractions were screened on polyacrylamide gel electrophoresis. As seen in Figure 1, the mutant and wild-type enzymes were all purified to homogeneity and exhibited molecular weights consistent with the established subunit weight of 45 000 (Bansal et al., 1989). The purified mutant glutamate dehydrogenases were analyzed for their N-terminal sequences. Each mutant enzyme exhibited the known sequence for *S. typhimurium* glutamate dehydrogenase, which has a cysteinyl residue in position five compared to a tyrosyl residue in the host *E. coli* enzyme (Bansal et al., 1989). These results demonstrate that the purified mutant enzymes were the glutamate dehydrogenase from *S. typhimurium*.

The specific activities for representative preparations at different stages of purification from crude extracts of wild-type and the mutant enzymes K286R, K286Q, and K286E are presented in Table I. These values were obtained using the substrate concentrations in the standard assay: 100 μM NADPH, 5 mM α-ketoglutarate, and 50 mM NH_4Cl. In order to evaluate the observed differences in specific activities, particularly the low activity measured for K286E (94 μmol of NADPH min^{-1} mg^{-1}) compared to wild-type enzyme (171 μmol of NADPH min^{-1} mg^{-1}), the K_m values for the individual substrates were determined.

Table I: Comparison of the Purification of Wild-Type and Mutant Glutamate Dehydrogenases[a]

enzyme	sp act. (μmol of NADPH min^{-1} mg^{-1}) crude extract	after Matrex Red column	after gel filtration
wild type	15.4	108	171
K286R	40.8	393	450
K286Q	47.8	174	252
K286E	17.6	87	94

[a] The specific activity of each enzyme preparation was determined in the crude extract, after Matrex Red chromatography, and after gel filtration by HPLC (TSK 4000 column). Activity was measured using the standard assay at 340 nm as described under Experimental Procedures.

Table II: $K_{m\text{-NADPH}}$ and k_{cat} for Wild-Type and Mutant Enzymes[a]

enzyme	K_m (μM)	k_{cat} (min^{-1}) I	II	av	$k_{cat}(av)/K_m$ (min^{-1} μM^{-1})
wild type	9.8	10.0×10^3	12.8×10^3	11.4×10^3	1.20×10^3
K286R	30	32.8×10^3	24.3×10^3	28.5×10^3	0.95×10^3
K286Q	66	20.6×10^3	18.5×10^3	19.6×10^3	0.30×10^3
K286E	280	19.5×10^3	18.0×10^3	18.8×10^3	0.07×10^3

[a] The assay solutions contained 50 mM potassium phosphate buffer (pH 8.0), 5 mM α-ketoglutarate, and 50 mM NH_4Cl. The concentration ranges of NADPH used were 2–100 μM for wild type and K286R, 16–400 μM for K286Q, and 28–1030 μM for K286E. The increased concentrations used for the K286Q and K286E enzymes required initial velocity measurements to be made at 375 nm rather than 340 nm. K_m and V_{max} were obtained from Lineweaver–Burk plots of $1/v$ vs $1/[NADPH]$. Duplicate experiments were averaged. $k_{cat}(I)$ was calculated from the extrapolated velocity estimated from saturating NADPH concentrations (micromoles of NADPH oxidized per minute per milligram of protein) and the molecular weight of the enzyme subunit; a minor correction for the concentration of α-ketoglutarate was made using the relationship $V_{max} = v[1 + (K_{m\text{-}\alpha KG}/[\alpha KG])$. The correction factors were as follows: for wild type, 1.06; for K286R, 1.14; for K286Q, 1.14; and for K286E, 1.21. $k_{cat}(II)$ was determined by utilizing equal concentrations of the enzymes at 2000 μM NADPH and assaying the activity of different enzymes in the same experiment at 390 nm.

Table III: Michaelis Constants for α-Ketoglutarate and NH_4Cl of Wild-Type and Mutant Glutamate Dehydrogenases[a]

enzyme	$K_{m\text{-}\alpha KG}$ (μM)	$K_{m\text{-}NH_4Cl}$ (mM)
wild type	270	3.6
K286R	680	3.7
K286Q	690	8.6
K286E	1000	5.5

[a] The apparent K_m values for α-ketoglutarate and NH_4Cl were determined from Lineweaver–Burk plots. The assays were at 25 °C in 50 mM potassium phosphate, pH 8. For the determination of $K_{m\text{-}\alpha KG}$, the substrate concentration was 100–2000 μM, with 50 mM NH_4Cl and levels of NADPH that were close to saturating (100 μM for wild type, 300 μM for K286R, 750 μM for K286Q, and 2000 μM for K286E). The wild-type and K286R mutant enzymes were assayed at 340 nm while the K286Q and K286E mutants were assayed at 390 nm. To determine $K_{m\text{-}NH_4Cl}$, the substrate concentration was 2–50 mM in the presence of 5 mM α-ketoglutarate and 2000 μM NADPH, and the assays were conducted at a wavelength of 390 nm.

Michaelis Constants for NADPH. Table II summarizes the apparent $K_{m\text{-NADPH}}$ (column 1) and $k_{cat}(I)$ (column 2) values for the mutant and wild-type enzymes. All three mutants exhibited elevated $K_{m\text{-NADPH}}$ values relative to that of wild type (K_m = 9.8 μM), although the apparent K_m for K286R (30 μM), which also contains a basic residue at position 286, exhibits the smallest change. Substitution of a neutral amino acid at position 286 in the K286Q enzyme resulted in a greater increase in $K_{m\text{-NADPH}}$ (66 μM). However, replace-

Table IV: Comparison of NADPH and NADH as Coenzymes for Wild-Type and Mutant Glutamate Dehydrogenases

	K_m[a] (μM)		k_{cat}[b] (min^{-1}) × 10^{-3}		k_{cat}/K_m (min^{-1} μM^{-1})		
enzyme	NADPH	NADH	NADPH	NADH	NADPH	NADH	NADPH/NADH
wild type	9.8	2000	11.3	1.22	1200	0.62	1900
K286R	30	1800	18.1	2.97	610	1.60	380
K286Q	66	2100	13.8	2.25	210	1.10	200
K286E	280	2100	11.8	0.95	42	0.46	91

[a] The K_m for NADPH is from Table II. The apparent K_m for NADH was determined in 50 mM potassium phosphate (pH 8.0) containing 50 mM NH_4Cl, 5 mM α-ketoglutarate, and 300–2000 μM NADH with assays at 390 nm. [b] Velocities [micromoles of NAD(P)H per minute per micromole of subunit] were obtained from reactions at 2000 μM coenzyme, 2 mM α-ketoglutarate, and 50 mM NH_4Cl and were extrapolated to V_{max} using the appropriate $K_{m\text{-NADPH}}$ or $K_{m\text{-NADH}}$ in the equation $V_{max} = v[1 + (K_{m\text{-NAD(P)H}}/2000\ \mu M)]$. These k_{cat} values were not extrapolated to saturating concentrations of α-ketoglutarate and are therefore different from those in Table II.

ment of the basic lysyl residue in the wild-type enzyme by the acidic glutamyl residue in the K286E mutant resulted in a 30-fold higher K_m for the coenzyme (280 μM). This observation is consistent with the concept that lysine-286 is normally involved in coenzyme binding. At the standard assay concentration of 100 μM NADPH, both the wild-type and K286R enzymes would have nearly saturating levels of coenzyme, but the mutant enzymes K286Q and K286E would not be saturated. The apparently low specific activity for the K286E mutant in Table I is, therefore, attributable to the nonsaturating NADPH concentration used.

As recorded in Table II, the k_{cat}(I) for K286R is 3 times that for wild-type enzyme, which is reflected in the high specific activity initially found (Table I). However, the efficiencies (k_{cat}/K_m) of the wild-type and K286R enzymes, both containing basic residues at position 286, are very similar: 1.2 × 10^3 min^{-1} μM^{-1} and 1.0 × 10^3 min^{-1} μM^{-1}, respectively (Table II, column 5). Although the efficiency of the K286Q mutant (with a neutral glutamine substitution) has been reduced, the efficiency of the K286E mutant (which has an acidic residue) is more than 16-fold lower than that of wild-type enzyme.

Michaelis Constants for α-Ketoglutarate (αKG). The $K_{m\text{-}\alpha KG}$ values for wild type and the mutants K286R, K286Q, and K286E were determined in phosphate buffer (50 mM, pH 8.0) with 50 mM NH_4Cl, over the αKG concentration range 100–2000 μM. In order to have saturating levels of coenzyme (Table II), the following concentrations of NADPH were used: for wild type, 100 μM; for K286R, 300 μM; for K286Q, 750 μM; and for K286E, 2000 μM. It was technically difficult to use a concentration of NADPH 10-fold higher than the K_m for the K286E mutant. The apparent $K_{m\text{-}\alpha KG}$ values obtained from Lineweaver–Burk plots are recorded in Table III. The mutants K286R and K286Q exhibited the similar values of 680 μM and 690 μM, showing only slightly weaker affinity for the substrate than does wild-type glutamate dehydrogenase (K_m = 270 μM). The mutant K286E had the highest $K_{m\text{-}\alpha KG}$ of 1000 μM, suggesting a small perturbation of substrate binding by this mutant enzyme. This slight increase in apparent $K_{m\text{-}\alpha KG}$ might reflect a local conformational change in the catalytic site due to the introduced negative charge.

Michaelis Constants for NH_4Cl. The $K_{m\text{-}NH_4Cl}$ experiments were performed in the presence of 100 μM and/or 2000 μM NADPH. Wild-type and K286R enzymes had apparent $K_{m\text{-}NH_4Cl}$ values between 3 and 9 mM at both coenzyme concentrations. The apparent $K_{m\text{-}NH_4Cl}$ values obtained with 2000 μM NADPH (used in order to saturate the K286E mutant) are given in Table III. These results indicate that substitution at position 286 had no appreciable effect on the affinity of enzyme for NH_4Cl.

Michaelis Constants for NADH. Although glutamate dehydrogenase is generally considered to be NADPH-specific, we were able to detect activity with NADH and to determine a $K_{m\text{-NADH}}$ for wild-type and mutant enzymes. The NADH concentrations used were 200–2000 μM and the activity was measured at 390 nm. Lineweaver–Burk plots were extrapolated to give the K_m values presented in Table IV (column 2). In view of the large extrapolation necessary, these apparent K_m values for NADH of wild-type and mutant enzymes are considered to be equivalent and demonstrate the great preference for NADPH by wild type and the three mutants at position 286 of glutamate dehydrogenase. The apparent $K_{m\text{-NADH}}$ for wild-type enzyme is 200 times greater than its $K_{m\text{-NADPH}}$. Although the acidic residue mutant K286E shows a 30-fold increase in $K_{m\text{-NADPH}}$ relative to wild-type enzyme, both enzymes have similar $K_{m\text{-NADH}}$ values.

Direct Comparison of NADPH and NADH as Coenzymes. The K_m values for NADH and NADPH reported above were determined in separate experiments with different amounts of the glutamate dehydrogenases. By measuring the enzyme activity of wild-type and mutant enzymes in the same experiment and at the same protein concentration in the presence of 2000 μM NADH or NADPH, a direct comparison of enzyme efficiency (k_{cat}/K_m) was made (Table IV).

The k_{cat} for wild-type enzyme using NADH as the coenzyme is $^1/_{10}$ that for NADPH (Table IV, columns 3 and 4). Furthermore, the k_{cat} measured with NADH is similar for all three mutants and wild-type enzyme (column 4). The efficiencies of the wild-type and mutant enzymes do not vary appreciably when NADH is used as coenzyme (column 6), indicating that none of the mutant enzymes exhibits an improved affinity for NADH. In comparison, a 27-fold decrease in efficiency is found with NADPH when the positively charged lysine-286 in wild-type enzyme is replaced by the negatively charged glutamyl residue in the K286E mutant (column 5). Thus, the higher NADPH/NADH efficiency ratio (column 7) of wild-type enzyme results from its lower $K_{m\text{-NADPH}}$ rather than an improved binding of NADH.

The conditions of this experiment permitted the separate determination of V_{max} for the NADPH reactions. These V_{max} values were in good agreement with those obtained in the individual $K_{m\text{-NADPH}}$ experiments and were used to calculate the k_{cat}(II) values. These k_{cat}(II) values are compared to the k_{cat}(I) values in Table II (column 3). An average of the two independently determined k_{cat} values was used to determine the efficiency reported in Table II, column 5.

Dependence of Velocity on pH for Wild-Type and Mutant Enzymes. The dependence of velocity on pH was initially measured under the standard assay conditions using 100 μM NADPH. As can be seen in Figure 2A, the optimum pH values observed for K286Q and K286E are lower (7.8 and 7.4, respectively) than that for the wild-type enzyme, pH 8.1–8.3. The mutant K286R as well as two additional mutants C283I and E284Q (available from our previous studies;

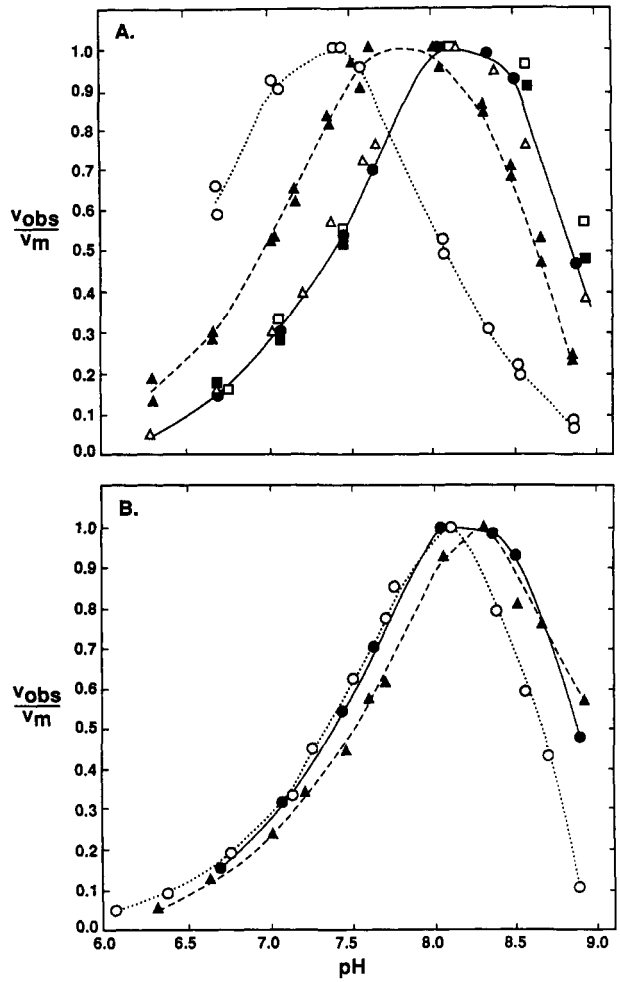

FIGURE 2: pH profiles of wild-type and mutant glutamate dehydrogenases. Each enzyme was assayed in a series of buffers; the final pH is plotted against a normalized velocity (the v_{obs} at a given pH divided by the maximum v_{obs} in that experiment). The ionic strength was maintained at 0.2 M as described in Experimental Procedures. (A) The assay solutions contained 100 μM NADPH, 50 mM NH_4Cl, and 5 mM α-ketoglutarate[2] in 50 mM potassium phosphate (pH 8). Representative experiments are shown for wild type (●), K286R (△), C283I (■), and E284Q (□). Duplicate experiments are shown for K286E (○) and K286Q (▲). (B) Concentrations of all substrates were the same as in (A) except that near saturating levels of NADPH were used for K286E (2000 μM; ○) and K286Q (1370 μM; ▲). Duplicate experiments were averaged for each mutant enzyme. Wild-type enzyme (●) was also analyzed at 1370 μM NADPH and is shown for comparison. The lines are not theoretical and are drawn by eye: [wild type (—), K286Q (- - -), and K286E (· · ·).

Haeffner-Gormley et al., 1991) all had pH profiles virtually identical to that of wild-type enzyme. The pH profiles of K286Q and K286E were repeated using concentrations of NADPH (1370 μM and 2000 μM, respectively) selected to be 10–20 times their corresponding K_m values (see Table II). Both K286Q and K286E under these conditions had pH profiles with optimum values of 8.1–8.3, similar to that of wild-type enzyme (Figure 2B). These results indicate that the different pH profiles observed in Figure 2A simply reflect the higher K_m values for the mutant enzymes, which may change with pH.

[2] The Lineweaver–Burk plots from the $K_{m\text{-}\alpha KG}$ experiments for wild-type and K286R glutamate dehydrogenase showed substrate inhibition with 5 mM α-ketoglutarate, the concentration used in the standard assay. To ensure that the pH profile studies of wild-type enzyme were not affected by the concentration of α-ketoglutarate, the pH profile was repeated with 2 mM α-ketoglutarate. The pH optimum was 8.09 and a pK_{aes} of 7.47 was found, confirming the original results.

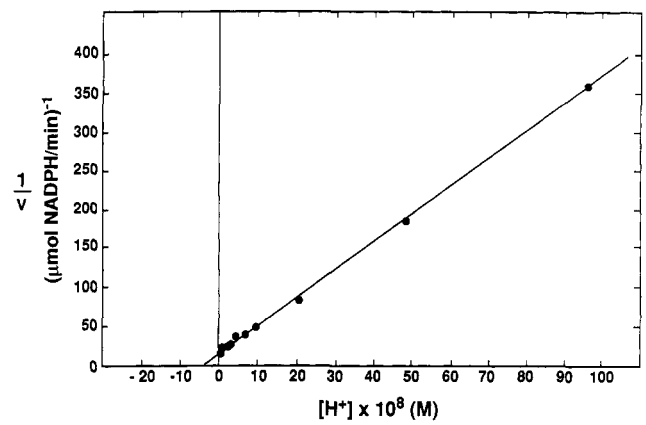

FIGURE 3: Determination of pK_{aes} for wild-type glutamate dehydrogenase from its pH profile. Wild-type enzyme was assayed using a series of buffers from pH 6.0 to 8.3 with constant ionic strength (0.2 M), 5 mM α-ketoglutarate, 50 mM NH_4Cl, and 100 μM NADPH. The final pH of each reaction was measured. The pK_{aes} was calculated from the slope of the line.[2]

It seemed likely that the plot of velocity versus pH for wild-type enzyme in Figure 2 represented the dependence of V_{max} on pH, at least over the pH range 6.0–8.3. The substrate concentrations were maintained constant at 100 μM NADPH, 5 mM α-ketoglutarate, and 50 mM NH_4Cl, levels high relative to their K_m values at pH 7.87 (the final pH in the standard assay): 9.8 μM for NADPH, 273 μM for α-ketoglutarate, and 3.58 mM for NH_4Cl. The K_m values of wild-type enzyme were determined separately at selected pHs. $K_{m\text{-NADPH}}$ did not vary in the pH range 6.72–8.10. $K_{m\text{-}NH_4Cl}$ was not appreciably different at pH 7.8 (3.6 mM) and pH 6.9 (9.4 mM), while $K_{m\text{-}\alpha KG}$ was decreased from 273 μM to 49 μM in this pH range. Therefore, substrate concentrations sufficient to saturate the wild-type enzyme at pH 7.87 would also be saturating at the lower pH values. The dependence of V_{max} of pH was analyzed in accordance with

$$V_{max} = \frac{V_{max_i}}{1 + \frac{[H^+]}{K_{aes}}} \quad (1)$$

where V_{max} is the maximum velocity observed at a given pH, V_{max_i} is the pH-independent maximum velocity, and K_{aes} is the ionization constant for the enzyme–substrate complex. The double reciprocal form of this equation is

$$\frac{1}{V_{max}} = \frac{1}{V_{max_i}} + \frac{[H^+]}{(V_{max_i})(K_{aes})} \quad (2)$$

A plot of $1/V_{max}$ vs $[H^+]$ allows the calculation of K_{aes} from the slope, as shown for a representative experiment in Figure 3. The average pK_{aes} for wild-type glutamate dehydrogenase was 7.52, suggesting the requirement for maximum activity of the unprotonated form of an ionizable group in the enzyme–substrate complex.

The dependence of V_{max} on pH for the mutant enzymes K286R, K286Q, and K286E obtained with NADPH concentrations of 100, 1350, and 2000 μM, respectively, were similarly analyzed, with the results shown in Table V. These results indicate that substitutions in the amino acid residue at position 286 do not appreciably influence the ionization of the group in the enzyme–substrate complex critical for maximum activity.

The observation that the pH profile of K286E shifted with the concentration of coenzyme (Figure 2) suggested that the

Table V: Determination of pK_{aes} from the pH Profile Studies[a]

enzyme	pK_{aes}
wild type	7.52
K286R	7.61
K286Q	7.64
K286E	7.49

[a] Enzyme assays were at 25 °C with buffers of different pH containing 50 mM NH_4Cl and 5 mM α-ketoglutarate. Near saturating levels of NADPH were used for each enzyme: wild type and K286R, 100 μM; K286Q, 1350 μM; and K286E, 2000 μM. The corresponding wavelengths used for assaying were 340 nm for wild type or K286R and 390 nm for K286Q or K286E. The pK_{aes} values from individual experiments were determined as illustrated for wild type in Figure 3. Similar plots for all the mutant enzymes were linear, indicating that these enzymes were saturated with substrates from pH 6 to 8. Average pK_{aes} values are given.

Table VI: Determination of $K_{m\text{-NADPH}}$ at Different pHs for Wild-Type and Mutant Glutamate Dehydrogenases[a]

enzyme	pH	$K_{m\text{-NADPH}}$ (μM)
wild type	6.72	6.5[b]
	7.40	6.7[b]
	8.10	10.8[b]
K286E	5.98	20.5[c]
	6.31	22.6[c]
	6.69	27.5[c]
	7.07	85.9[c]
	7.38	171[c]
	7.58	289[d]
	8.08	850[d]
K286Q	6.30	12.5[b]
	6.69	19.4[b]
	7.02	19.3[b]
	7.38	18.2[b]
	7.57	39.3[c]
	8.08	80.1[c]

[a] $K_{m\text{-NADPH}}$ was determined from Lineweaver–Burk plots. The different buffers contained 5 mM α-ketoglutarate, 50 mM NH_4Cl, and various concentrations of NADPH; the ionic strength was maintained at 0.2 M. Duplicate experiments were averaged. [b] Assays were conducted at 340 nm. [c] Assays were conducted at 375 nm. [d] Assays were conducted at 390 nm.

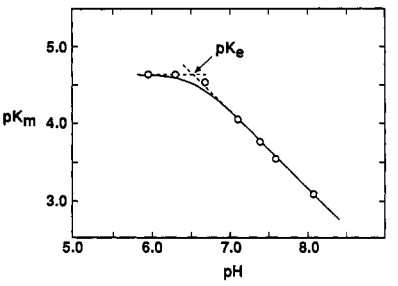

FIGURE 4: Plot of $pK_{m\text{-NADPH}}$ vs pH for K286E mutant. The $K_{m\text{-NADPH}}$ for the K286E mutant is essentially constant below pH 6.3. Above pH 7, the pK_m vs pH plot exhibits a slope of −1.0. This line is extrapolated to intersect with the plateau region of pK_m at the lower pH values to yield an estimated pK_e of 6.5 (dashed line) (Dixon & Webb, 1964).

$K_{m\text{-NADPH}}$ varied with pH. Table VI records the change in $K_{m\text{-NADPH}}$ over the pH range 6–8. $K_{m\text{-NADPH}}$ decreases more than 30-fold for the K286E enzyme as the pH is decreased from 8.1 to 6.7, whereas the wild-type enzyme shows little change in the $K_{m\text{-NADPH}}$ over the same pH range. In the case of the K286E mutant enzyme, the K_m values obtained at the different pHs were also plotted as pK_m vs pH (Figure 4). A pK of 6.5 was estimated. This plot cannot in itself distinguish between ionizations of the free enzyme and free substrate (Dixon & Webbs, 1964). However, the free substrate NADPH is known to exhibit, under comparable conditions, a pK of 6.13 for the 2′-phosphate (Mas & Colman, 1984). Therefore, the pK of 6.5 is probably due to the ionization of the free enzyme and can be designated pK_e.

The $K_{m\text{-NADPH}}$ was also determined as a function of pH for the mutant K286Q enzyme. A 4-fold decrease in K_m was found as the pH was lowered from 8.1 to 6.7 (Table VI). These changes are too small to reflect directly the dependence of $K_{m\text{-NADPH}}$ on the protonation of a single ionizable group with a pK in this pH range and thus the results for the K286Q mutant contrast with those for the K286E mutant.

Kinetic Studies on the K278Q Mutant of Glutamate Dehydrogenase. In *S. typhimurium* glutamate dehydrogenase, another lysine residue is located at position 278, which is also close to the peptide 282–286 implicated by affinity labeling as the region of the coenzyme binding site (Haeffner-Gormley et al., 1991, 1992a). A possible role for lysine-278 in the coenzyme binding site was investigated using the mutant K278Q enzyme. The kinetic studies were performed in a manner analogous to those on the wild-type enzyme. The following results were obtained: $K_{m\text{-NADPH}} = 10.3$ μM, $K_{m\text{-αKG}} = 456$ μM, and $K_{m\text{-NH}_4Cl} = 2.38$ mM. A k_{cat} value of 10.5×10^3 min^{-1} and an efficiency (k_{cat}/K_m) of 1.02×10^3 min^{-1} μM^{-1} were determined. The pH optimum was 8.39, with a pK_{aes} of 7.38. All these values are similar to those of wild-type enzyme and indicate that lysine-278 is not associated with the binding of the 2′-phosphate of NADPH.

DISCUSSION

The replacement of lysine-286 in glutamate dehydrogenase from *S. typhimurium* by residues that were basic, neutral, or acidic resulted in enzymes that were catalytically competent. The three mutant enzymes, K286R, K286Q, and K286E, were readily purified by the same method as was the wild-type enzyme using a dye-ligand column. These observations indicated that no gross conformational change of the enzyme or its binding site had occurred.

The nucleotide binding fold in a number of enzymes has been characterized by a region containing a βαβ configuration (Wierenga et al., 1985). Among the NADP enzymes, dihydrofolate reductase (DHFR) and glutathione reductase have been studied in detail. Although the nucleotide fold of DHFR has some unique features, both DHFR and glutathione reductase illustrate the interaction of basic groups in the protein with the 2′-phosphate group of the coenzyme. In DHFR from *Lactobacillus casei* arginine-43 interacts with the 2′-phosphate group (Filman et al., 1982). This arginine residue is conserved in most DHFR enzymes but is replaced by lysine in chicken and human DHFR (Birktoft & Banaszak, 1984). The mutation of this arginine residue (R44L) in *E. coli* DHFR disrupted the ionic contact with the coenzyme's 2′-phosphate group (Adams et al., 1989), and the mutation of the corresponding lysine (K54Q) in human DHFR produced an enzyme with a 58-fold higher $K_{m\text{-NADPH}}$. In the case of human erythrocyte NADP-glutathione reductase, three basic amino acids contribute to the binding site of the 2′-phosphate of the coenzyme (Pai et al., 1988). The glutathione reductase from *E. coli* has a lysine in place of a histidine in the human enzyme, and the mutation of one arginine substantially decreased the affinity of the *E. coli* enzyme for NADPH (Scutton, 1990).

Glutamate dehydrogenase from *E. coli* has been predicted to be a member of the group of enzymes with the βαβ motif for binding coenzyme (Kuroda et al., 1989). By analogy, the enzyme from *S. typhimurium* would also be expected to be

in this subclass of enzymes, all of which have an association of one or more basic groups with NADPH binding. The hypothesis that lysine-286 plays a role in NADPH binding was here confirmed by the $K_{m\text{-NADPH}}$ determinations for the mutant enzymes. The mutant K286E has a significantly higher K_m than the wild-type or K286R enzymes, indicating that the change in charge interferes with coenzyme binding. Furthermore, the mutant K286R, which preserves the positive charge, has an efficiency (1.0×10^3 min^{-1} μM^{-1}) similar to that of wild-type enzyme (1.20×10^3 min^{-1} μM^{-1}). The K286Q mutant, contributing no charge to the region, exhibits an intermediate $K_{m\text{-NADPH}}$, indicating that a basic amino acid at position 286 tightens the binding but is not an absolute requirement either for NADPH binding or for catalysis. The decrease in affinity for NADPH in the K286E mutant could result directly from the loss of the lysyl residue positive charge or from a local disruption in conformation, perhaps due to a charge repulsion. The former postulate seems likely in view of the low $K_{m\text{-NADPH}}$ of the K286R mutant. Electron density studies of DHFR show that a polarization is induced in the coenzyme NADPH upon binding to the enzyme, in addition to more local electrostatic interactions with the critical basic amino acids (Bajorath et al., 1991). The question of whether the replacement of lysine by glutamic acid disrupts the local electrostatic interaction with the 2′-phosphate or whether it also interferes with an overall polarization of the bound NADPH molecule (as suggested by the DHFR study) will probably remain unresolved until a three-dimensional structure of NADP-glutamate dehydrogenase is known. Further support for the importance of a basic residue at position 286 is evident in this residue's conservation in the NADP-glutamate dehydrogenase enzymes from *E. coli* and *Neurospora crassa*. Moreover, the corresponding residue is alanine in the NAD-glutamate dehydrogenase from *Clostridium symbiosum*, which also has the $\beta\alpha\beta$ nucleotide fold (Rice et al., 1987; Baker et al., 1992).

Although the mutations at position 286 produce changes in the apparent $K_{m\text{-NADPH}}$, all three mutants and the wild-type enzyme have k_{cat} values of the same order of magnitude. Therefore, the altered efficiencies (k_{cat}/K_m) of the enzymes reflect primarily changes in the $K_{m\text{-NADPH}}$ values. Replacement of the positive lysine residue by glutamic acid produces an enzyme which has a 16-fold lower efficiency.

In the case of the K286E enzyme, but not the wild-type enzyme, the $K_{m\text{-NADPH}}$ decreases markedly with decreasing pH (Table VI). A pK_e of 6.5 was calculated for the K286E mutant enzyme from these determinations. This pK of the free mutant enzyme could represent an abnormally high pK for the carboxyl group of the glutamic acid replacement, which may reflect either proximal negative charge(s) or a hydrophobic environment for the residue at position 286. The $K_{m\text{-NADPH}}$ of the mutant K286Q also decreases somewhat with pH, but the change is too small to be attributable to a single ionizable group with a pK in the pH range 6.1–8.1.

When saturating levels of substrates (including NADPH) are present, all the position 286 mutants and wild-type enzyme exhibit pH optima of 8.1–8.3. The pH optimum for wild-type glutamate dehydrogenase from *E. coli* is 8.5 (McPherson et al., 1988), very close to the value obtained with *S. typhimurium*, as would be expected on the basis of the similarity of these two bacterial NADP enzymes. Furthermore, the wild-type and all three mutant enzymes exhibit pK_{aes} values of 7.5–7.6. Glutamate dehydrogenase from *E. coli* has a reactive lysine residue with an unusually low pK_{aes} of 7.6 (McPherson et al., 1988). This residue was identified as lysine-128 (equivalent to lysine-128 in *S. typhimurium*) and was associated with substrate binding. The present study indicates that substitution for residue 286 in the *S. typhimurium* enzyme does not affect the low pK_{aes} which is presumably attributable to lysine-128.

Although glutamate dehydrogenase from *S. typhimurium* and *C. symbiosum* are respectively NADP$^+$- and NAD$^+$-specific enzymes, there appears to be a significant homology between them (Baker et al., 1992). It was, therefore, of interest to see if our residue 286 mutants demonstrated any improved capacity to bind NADH. The three mutants, K286R, K286Q, and K286E, exhibit $K_{m\text{-NADH}}$ values similar to that of wild-type enzyme, although the K286E mutant has a greatly increased $K_{m\text{-NADPH}}$ (Table IV). This result is similar to that found for the dihydrofolate reductase mutant K54Q, where the $K_{m\text{-NADPH}}$ is increased, but the $K_{m\text{-NADH}}$ of the mutant and wild-type DHFR are of the same order of magnitude (Huang et al., 1990).

It is likely that more than one basic amino acid of glutamate dehydrogenase interacts with the 2′-phosphate of NADPH. However, our studies with the K278Q mutant indicate that lysine-278 is not important for coenzyme binding. This observation is consistent with the fact that lysine-278 is not conserved in all comparable glutamate dehydrogenases: in the *N. crassa* enzyme, an aspartic acid appears at the equivalent position, while in the NAD-glutamate dehydrogenase from *C. symbiosum*, a basic arginine appears at the corresponding position. Once the X-ray structure of NADP-glutamate dehydrogenase is available other basic amino acids may be recognized as having possible coenzyme binding roles.

The evidence in the present study is consistent with the concept that the basic residue lysine-286 has a role in the binding of the 2′-phosphate group of NADPH in glutamate dehydrogenase from *S. typhimurium*. This investigation adds support to the generalization of such interactions predicted by studies of other NADP$^+$ enzymes.

ACKNOWLEDGMENT

We thank Dr. David Rice (University of Sheffield, UK) for providing prior to publication the X-ray crystallographic results for NAD$^+$-glutamate dehydrogenase from *C. symbiosum*. We also thank Dr. Yu-Chu Huang for the amino acid sequence analyses.

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Registry No. Glu, 56-86-0; Arg, 74-79-3; Gln, 56-85-9; Lys, 56-87-1; NADH, 58-68-4; $NADP^+$-glutamate dehydrogenase, 9029-11-2.