Properties of Lipoamide Dehydrogenase Altered by Site-Directed Mutagenesis at a Key Residue (I184Y) in the Pyridine Nucleotide Binding Domain†

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ABSTRACT: The binding of pyridine nucleotide to human erythrocyte glutathione reductase, an enzyme of known three-dimensional structure, requires some movement of the side chain of Tyr^{197}. Moreover, this side chain lies very close to the isoalloxazine ring of the FAD cofactor. The analogous residue, Ile^{184}, in the homologous enzyme *Escherichia coli* lipoamide dehydrogenase has been altered by site-directed mutagenesis to a tyrosine residue (I184Y) [Russell, G. C., Allison, N., Williams, C. H., Jr., & Guest, J. R. (1989) *Ann. N.Y. Acad. Sci. 573*, 429–431]. Characterization of the altered enzyme shows that the rate of the pyridine nucleotide half-reaction has been markedly reduced and that the spectral properties have been changed to mimic those of glutathione reductase. Therefore, Ile^{184} is shown to be an important residue in modulating the properties of the flavin in lipoamide dehydrogenase. Turnover in the dihydrolipoamide/NAD^+ reaction is decreased by 10-fold and in the NADH/lipoamide reaction by 2-fold in I184Y lipoamide dehydrogenase. The oxidized form of I184Y shows remarkable changes in the fine structure of the visible absorption and circular dichroism spectra and also shows nearly complete quenching of FAD fluorescence. The spectral properties of the altered enzyme are thus similar to those of glutathione reductase and very different from those of wild-type lipoamide dehydrogenase. On the other hand, spectral evidence does not reveal any change in the amount of charge-transfer stabilization at the EH_2 level. Stopped-flow data indicate that, in the reduction of I184Y by NADH, the first step, reduction of the flavin, is only slightly slowed but the subsequent two-electron transfer to the disulfide is markedly inhibited. This allows direct observation of the presumed reduced flavin species. Reduction of I184Y by dihydrolipoamide to the EH_2 stage is not detectably altered, as expected given the site of the alteration.

Dihydrolipoamide dehydrogenase (EC 1.8.1.4), a member of the FAD-containing pyridine nucleotide–disulfide oxidoreductase family, catalyzes the reaction:[1]

$$Lip(SH)_2NH_2 + NAD^+ \rightleftarrows LipS_2NH_2 + NADH + H^+$$

Among the other members of this family, which include glutathione reductase, mercuric reductase, and thioredoxin reductase, glutathione reductase is the most thoroughly investigated both structurally and mechanistically. Physiologically, lipoamide dehydrogenase catalyzes the reduction of pyridine nucleotide while glutathione reductase catalyzes pyridine nucleotide oxidation. However, lipoamide dehydrogenase catalyzes the thermodynamically favorable pyridine nucleotide oxidation by lipoamide with great efficiency at neutral pH (Massey et al., 1960). These enzymes, therefore, have distinct catalytic roles, suggesting that the amino acid residues moderating the redox properties of the FAD and the redox-active disulfide operate slightly differently for each enzyme.

The structure of glutathione reductase from human erythrocytes has been analyzed by X-ray crystallography to a resolution of 0.154 nm, allowing the active site to be defined in great detail (Thieme et al., 1981; Pai & Schulz, 1983; Karplus & Schulz, 1987). The functions of specific amino acid residues have been assigned in structural and in mechanistic studies (Pai & Schulz, 1983; Karplus & Schulz, 1987; Arscott et al., 1981; Thorpe & Williams, 1976a; Matthews & Williams, 1976). One of the interesting residues identified in the structural study is Tyr^{197} in the NADPH binding site of human erythrocyte glutathione reductase. It is seen that the phenol ring of Tyr^{197} must move aside in order for the pyridinium ring of NADPH to stack with the isoalloxazine ring of the FAD (Pai & Schulz, 1983). Indeed, alteration of this residue leads to an apparent change from ping-pong to sequential kinetics and lessens the quenching of the flavin fluorescence (Berry et al., 1989). This tyrosine residue is conserved in glutathione reductase from human erythrocytes and *Escherichia coli* (Krauth-Siegel et al., 1982; Greer & Perham, 1986) but replaced by Ile or Val in lipoamide dehydrogenase from *E. coli*, *Azotobacter vinelandii*, yeast, pig heart, and human liver (Stephens et al., 1983; Westphal & de Kok, 1988; Browning et al., 1988; Ross et al., 1988; Otulakowski & Robinson, 1987; Pons et al., 1988). It seemed likely that the presence or absence of this aromatic ring would influence the properties of the adjacent flavin ring. The crystal structure of *A. vinelandii* lipoamide dehydrogenase at the resolution currently available suggests that the important residues are similarly disposed within the immediate active site (Schierbeek et al., 1989).

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[1] Abbreviations: I184Y, site-directed mutation of lipoamide dehydrogenase with isoleucine-184 replaced with a tyrosine residue; $Lip(SH)_2NH_2$, dihydrolipoamide; $LipS_2NH_2$, lipoamide; APADH and $APAD^+$, reduced and oxidized forms of acetylpyridine adenine dinucleotide, respectively; AAD^+, aminopyridine adenine dinucleotide.

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Guest and his colleagues (Allison et al., 1988; Williams et al., 1989; Russell et al., 1989) have used site-directed mutagenesis to alter several residues of interest in *E. coli* lipoamide dehydrogenase. In one of the mutants, Ile^{184} is replaced by Tyr (I184Y). In this paper, we report the dramatic effects of this alteration on the enzymatic activities and spectral characteristics of the oxidized and reduced forms of I184Y and compare it with wild-type lipoamide dehydrogenase and glutathione reductase.

Experimental Procedures

Reagents. 3-Aminopyridine adenine dinucleotide was synthesized by the method of Fisher et al. (1973). Deazaflavin (7,8-didemethyl-N^3-methyl-5-deazalumiflavin) was the gift from the late Dr. P. Hemmerich. $Lip(SH)_2NH_2$ and $LipS_2NH_2$ were purchased from Farmochimica Cutolo-Calosi, bactotryptone and bacto yeast extract were from DIFCO Laboratories, Q-Sepharose was from Pharmacia, potassium phosphate monobasic, potassium phosphate dibasic, and EDTA were from Fisher Scientific, and other reagents were from Sigma.

Mutagenesis and Growth of the Cells. Site-directed mutagenesis of the *E. coli* lipoamide dehydrogenase was carried out as previously described (Allison et al., 1988; Williams et al., 1989; Russell et al., 1989). Because the host *E. coli* strain (JRG1342 Δ*ace-lpd*) lacks the gene for lipoamide dehydrogenase, there was no native enzyme present unless the cells were transformed with plasmid containing the gene for the native enzyme. Plasmid-containing strains of *E. coli* were grown at 30 °C in an enriched medium, Terrific Broth (Tartof & Hobbs, 1988) with ampicillin (25 μg/mL) and glucose (0.5%) on a Petri dish (2 days), in 6 × 3 mL cultures (overnight), in 6 × 25 mL cultures (overnight), and in 6 × 1 L cultures. Before the 1-L cultures were started, Antifoam B was added (0.1 mL/L). The cultures at the 1-L stage were rapidly shifted to 42 °C when the absorbance at 650 nm reached 0.4–0.5 and then incubated at 42 °C for 4 h. Cells were harvested by centrifugation at 8000 rpm for 10 min, frozen with liquid nitrogen, and stored at −20 °C.

Enzyme Purification. Mutant and wild-type lipoamide dehydrogenases were purified by a simplified procedure applicable to both. Frozen cells were thawed, homogenized in 0.1 M phosphate buffer, pH 7.6, containing 0.3 mM EDTA and 4.5 mg/L phenylmethanesulfonyl fluoride, and sonified for five bursts of 3 min each using a Branson Sonifier, Model 200, in a rosette cell immersed in an ice–NaCl–water mixture. Nucleic acids were precipitated by the addition of streptomycin sulfate to 2.5% (w/v) and removed together with cellular debris by sedimentation in a Beckman Model L8-70M ultracentrifuge using a VTi-50 rotor at 25 000 rpm for 20 min and then at 45 000 rpm for 1 h. The supernatant was fractionated with ammonium sulfate from 40% to 75% saturation, and the precipitate was dialyzed overnight against 0.02 M phosphate buffer containing 0.3 mM EDTA and 4.5 mg/L phenylmethanesulfonyl fluoride, pH 7.6. After a brief centrifugation, the dialysate was fractionated at room temperature by FPLC on a Q-Sepharose column. The column was washed with 0.02 and then 0.1 M buffer exhaustively. Elution of lipoamide dehydrogenase was effected by making the latter buffer 0.1 M in KCl. If necessary, this chromatography step was repeated. Enzymes were stored in 0.1 M potassium phosphate buffer, pH 7.6, containing 0.3 mM EDTA at 4 °C, and this buffer was used in all experiments except where otherwise noted. About 200 mg of native lipoamide dehydrogenase or I184Y was obtained from 20 g (wet weight) of cells. The purity of the enzymes was established by the spectral ratio between 280 nm and the FAD peak position (5.8 for wild-type enzyme and 6.7 for I184Y).

Spectral Measurements. The concentration of enzyme samples was determined using an extinction coefficient of 11.3 mM^{-1} cm^{-1} for the enzyme-bound FAD in I184Y (462 nm) and wild-type (455 nm) lipoamide dehydrogenases. The concentration of thiols was determined by titration with 5,5′-dithiobis(2-nitrobenzoic acid) (Thorpe & Williams, 1974). The concentrations of NAD^+, NADH, and their analogues were determined from their absorption spectra.

Enzymatic assays at 340 nm contained 80 μM $Lip(SH)_2NH_2$ and 100 μM NAD^+ in 0.1 M phosphate buffer, pH 7.6, containing 0.3 mM EDTA (25 °C). Absorption spectra were recorded with a Cary 219 or Hewlett-Packard 8452A diode array spectrophotometer with temperature control. Fluorescence spectra were recorded at 25 °C with a ratio spectrofluorometer built by Mr. Gordon Ford and Dr. David P. Ballou at the University of Michigan. Circular dichroism spectra were measured with a JASCO J-40C spectrometer and a Tracor Northern Digital signal analyzer, TN-1500. Rapid reaction kinetics were measured with a stopped-flow spectrophotometer interfaced with a Nova 2 (Data General) minicomputer (Beaty & Ballou, 1981).

Sample solutions for anaerobic experiments were prepared by sequential cycling between vacuum and oxygen-free argon gas (at least ten times), the final phase being equilibration with argon gas. High-purity argon gas was pretreated by passage through an oxygen trap (R & D Separations). Photoreduction was in the presence of EDTA and a catalytic amount of 5-deazaflavin as described previously (Massey & Hemmerich, 1978).

Results

Enzymatic Activities of Wild-Type and I184Y Lipoamide Dehydrogenases from E. coli. Wild-type lipoamide dehydrogenase shows strong product inhibition by NADH on the physiological activity, $Lip(SH)_2NH_2$–NAD^+ (Wilkinson & Williams, 1981). With enzyme concentrations of 0.2–0.5 nM, it is possible to determine the steady-state kinetic parameters of wild-type lipoamide dehydrogenase from *E. coli* and to compare these with the I184Y enzyme. Lineweaver–Burk plots of wild-type lipoamide dehydrogenase indicated a bi-bi ping-pong mechanism with a turnover number of 770 mol s^{-1} (mol of FAD)$^{-1}$, a K_m value for $Lip(SH)_2NH_2$ of 70 μM, and a K_m value for NAD^+ of 400 μM at pH 7.6 and 25 °C. Thus, this turnover number is somewhat higher than that determined in a rapid-reaction study (420 s^{-1}, pH 7.5) (Sahlman & Williams, 1989), but is almost the same as that of the pig heart enzyme (Massey et al., 1960; Matthews et al., 1977, 1979). At high concentrations of both substrates relative to the K_m values of wild-type lipoamide dehydrogenase, I184Y showed the same pH dependence of the activity but the turnover number was decreased to 9% (70 s^{-1}) of that of the wild-type enzyme at the optimal pH of 7.6. Lineweaver–Burk plots of I184Y showed strong inhibition by the electron donor $Lip(SH)_2NH_2$ at concentrations above 20 μM, and the degree of inhibition depended on the electron-acceptor concentration between 0.1 and 2.0 mM NAD^+. From a Dixon plot, $Lip(SH)_2NH_2$ behaves as an apparent competitive inhibitor to NAD^+ with a K_i value of 20 μM at pH 7.6 and 25 °C.

NAD^+ acts as both an activator and inhibitor in the reverse reaction, NADH–$LipS_2NH_2$ activity, of wild-type lipoamide dehydrogenase, making it difficult to determine the steady-state parameters (Wilkinson & Williams, 1981). With 80 μM NADH and 1.3 mM $LipS_2NH_2$ at 25 °C and pH 7.9, the

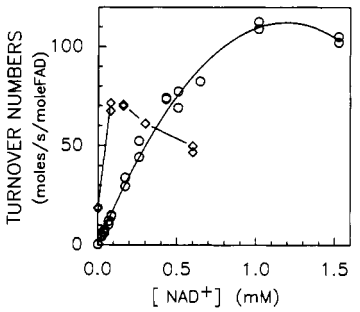


FIGURE 1: Effect of NAD^+ on NADH–$LipS_2NH_2$ activity of I184Y and wild-type lipoamide dehydrogenases. Assay concentrations were 80 μM NADH and 1.3 mM $LipS_2NH_2$ in 0.1 M potassium phosphate buffer, pH 7.9, at 25 °C. Reactions were started by adding enzyme. Final enzyme concentrations were 3 and 2 nM for I184Y lipoamide dehydrogenase (◇) and wild-type lipoamide dehydrogenase (○), respectively.

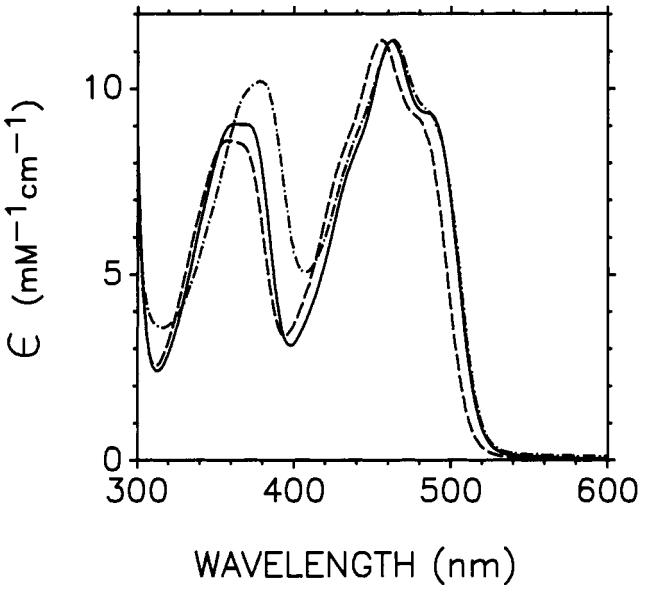


FIGURE 2: Absorption spectra of I184Y and wild-type lipoamide dehydrogenases and glutathione reductase. Spectra of 0.17 mM I184Y lipoamide dehydrogenase (—), 0.22 mM wild-type lipoamide dehydrogenase (---), and 0.17 mM yeast glutathione reductase (–·–) were taken in 0.1 M potassium phosphate buffer, pH 7.6, containing 0.3 mM EDTA at 4 °C. The cell path length was 2mm.

turnover number of wild-type lipoamide dehydrogenase increased from 0 to 110 mol s^{-1} (mol of FAD)$^{-1}$ by adding NAD^+ from 0 to 1 mM and then decreased with more NAD^+ (Figure 1), results comparable to a previous study (Wilkinson & Williams, 1981). On the other hand, I184Y had some activity without addition of NAD^+ [18 mol s^{-1} (mol of FAD)$^{-1}$] and showed a maximum turnover number of 67 mol s^{-1} (mol of FAD)$^{-1}$ with 20 μM NAD^+ (Figure 1). The optimal pH of 7.6 for this reverse reaction of I184Y with 20 μM NAD^+ was the same as that of wild-type lipoamide dehydrogenase with 1 mM NAD^+. Partial inhibition by the electron donor was also observed at concentrations over 10 μM NADH in the absence of NAD^+.

Spectral Properties of the Oxidized Enzyme Form. The addition of a tyrosine residue in I184Y results in the expected increase in the spectral ratio between 280 nm and the FAD peak position from 5.8 in the wild-type enzyme to 6.7 in I184Y. The absorption spectrum of I184Y in the visible wavelength region is shown in Figure 2, with those of wild-type lipoamide dehydrogenase from *E. coli* and human erythrocyte glutathione reductase. The spectral shape is not changed by the mutation, but the absorption maximum of I184Y is at 462 nm, a red shift

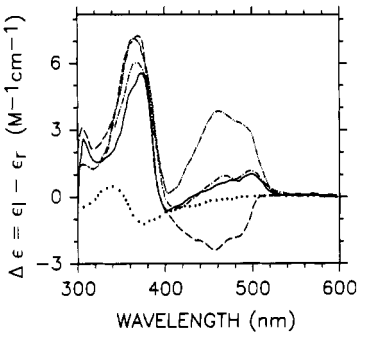


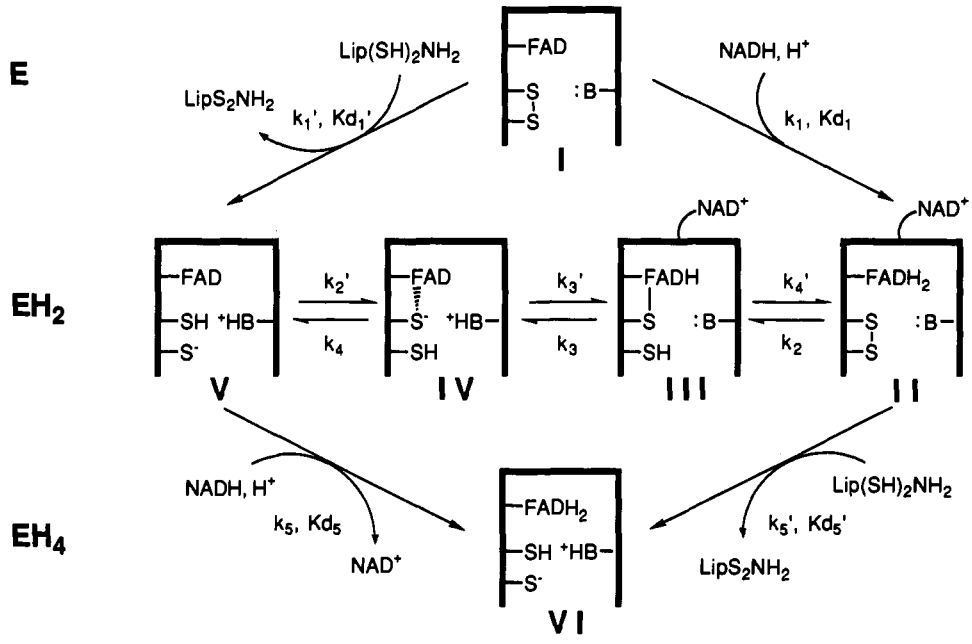
FIGURE 3: Circular dichroism spectra of I184Y and wild-type lipoamide dehydrogenases, pure FAD, and glutathione reductase. Spectra of 0.17 mM I184Y (—), 0.22 mM wild-type lipoamide dehydrogenase (---), 0.20 mM pure FAD (···), 0.17 mM yeast glutathione reductase (–·–), and human erythrocyte (–··–) glutathione reductase were taken in 0.1 M potassium phosphate buffer, pH 7.6, containing 0.3 mM EDTA at 4 °C except that the glutathione reductase was at 25 °C. The spectral data were the average of three scans.

of 7 nm from that of wild-type lipoamide dehydrogenase, and is virtually identical with that of glutathione reductase from yeast and human erythrocytes. From the second and fourth derivatives of the absorption spectra (Butler, 1972), vibronic transitions are found at 488, 455, 428, 403, 374, 353, and 336 nm for wild-type lipoamide dehydrogenase and 495, 462, 432, 408, 378, 356, and 338 nm for I184Y. Thus, there is a parallel shift of the vibronic sublevels of each absorption band between wild-type and I184Y lipoamide dehydrogenases. For comparison, human erythrocyte glutathione reductase has vibronic transitions at 496, 463, 435, 414, 386, 365, and 344 nm. Neither pH change between 6.0 and 8.5 nor binding of the oxidized form of the substrates perturbs the FAD spectrum of I184Y lipoamide dehydrogenase.

The circular dichroism spectrum in the visible wavelength region is also changed by the I184Y mutation (Figure 3). Wild-type lipoamide dehydrogenase shows negative and positive Cotton effects associated with the visible and near-ultraviolet absorption bands, respectively. These circular dichroism bands show fine structure, and each peak position corresponds with those of the absorption spectra. Although lipoamide dehydrogenases from pig heart and from wild-type *E. coli* show the same absorption spectra in the visible and near-ultraviolet wavelength regions, the pig heart enzyme shows no circular dichroism associated with the visible absorption band and has a 3-fold larger positive circular dichroism associated with the near-ultraviolet absorption band. In I184Y, two positive circular dichroism bands and one negative circular dichroism band are observed in the visible absorption region, and a less intense positive circular dichroism band, compared to wild type, is observed in the near-ultraviolet absorption band. Thus, the circular dichroism of I184Y between 300 and 500 nm is very similar to that of yeast glutathione reductase (Figure 3). The circular dichroism of I184Y has a sharp negative band at 280 nm, which indicates that the new tyrosine residue is in a restricted position (not shown). However, the far-ultraviolet circular dichroism of wild-type lipoamide dehydrogenase and I184Y is virtually identical, indicating the mutation causes no change in the overall structure of the protein.

The I184Y mutation induces a dramatic change in the fluorescence of the enzyme-bound FAD. While the FAD of both lipoamide dehydrogenases from pig heart and wild-type *E. coli* shows high fluorescence (5.6–5.7 times that of free FAD; excitation at 450 nm, 4 °C, and pH 7.6), the fluorescence of FAD of I184Y is only 3% that of free FAD. This

Scheme I: Pathways of Reduction of *E. coli* Lipoamide Dehydrogenase by Its Substrates



almost complete fluorescence quenching is similar to that seen in glutathione reductase.

Spectral Properties of the Two-Electron- and Four-Electron-Reduced Enzyme Forms (EH_2 and EH_4). I184Y and wild-type lipoamide dehydrogenases show the same spectral changes upon reduction with ~5-fold excess of $Lip(SH)_2NH_2$, NADH, NADPH, or APADH, under anaerobic conditions. After rapid formation of the charge-transfer-type EH_2 species, both enzymes are reduced to EH_4 and showed similar EH_4 spectra with a peak at 420 nm. A 7-fold excess of APADH (even with a redox potential 60 mV higher than that of NADH) reduces both I184Y and wild-type lipoamide dehydrogenases to EH_4 at pH 7.0, but these reductions at pH 7.6 are not complete. Photoreduction of wild-type lipoamide dehydrogenase and I184Y results in the sequential formation of the neutral semiquinone, the charge-transfer complex (EH_2), and EH_4 (Figure 4). The character of the EH_4 spectra is pH dependent. At pH 7.6, the EH_4 spectrum of I184Y has a peak around 420 nm, indicating the neutral reduced flavin form, but that of the wild-type enzyme lacks this peak, indicating the anionic form of reduced flavin (Walker et al., 1970). At pH 8.5, neither EH_4 enzyme shows a peak at 420 nm, and both show the peak at pH 6.0. Although exact pK values have not been determined, it is clear that the pK_a value of the N-1 protonation of reduced FAD in I184Y is somewhat higher than in wild-type lipoamide dehydrogenase.

During the reoxidation of EH_4 by air in the presence of NAD^+, both enzymes show a transient, broad band centered at 750 nm. A similar band, seen when wild-type *E. coli* or pig heart enzyme is reduced with NADH in the presence of arsenite and excess NAD^+, or slowly when the temperature of the NADH-reduced enzyme was lowered, was attributed to charge transfer from enzyme-bound $FADH_2$ to NAD^+ (Massey & Palmer, 1962; Williams, 1965). This band is not observed with enzyme at the EH_4 level in the presence of NADPH, $NADP^+$, AAD^+, APADH, $APAD^+$, or $Lip(SH)_2NH_2$.

Stopped-Flow Measurements. The reduction of wild-type lipoamide dehydrogenase by NADH is very fast. Reduction of the FAD (Scheme I, k_1) and transfer of electrons to the active-site disulfide (k_2, k_3) are essentially complete in the 3-ms dead time of the rapid reaction spectrophotometer. Reduction of the FAD in I184Y is also very fast, but the subsequent electron transfer to the disulfide is much slower in this altered enzyme. Thus, the reduced flavin species, species II in Scheme I, can be observed. The kinetics for the first 100 ms of this reaction are compared in Figure 5 for the I184Y and wild-type enzyme. FAD reduction is observed at 455 (or 462) nm, the $FADH_2$ to NAD^+ charge-transfer complex at 719 nm (species II), and the thiolate to FAD charge-transfer complex at 535 nm (species IV). With the wild-type enzyme, FAD reduction and reoxidation of the flavin within the EH_2 species II–V are

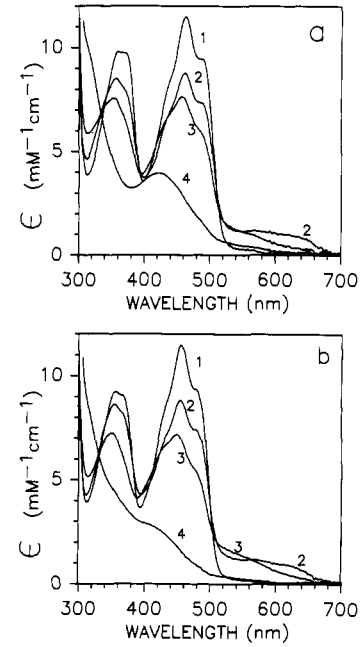


FIGURE 4: Photoreduction of I184Y and wild-type lipoamide dehydrogenases. Photoreductions were carried out under anaerobic conditions at pH 7.6 in the presence of 5 μM deazaflavin and 25 mM EDTA at 4 °C. (a) Curves 1–4, before and after 21, 46, and 180 min of illumination, respectively, of 14.1 μM I184Y lipoamide dehydrogenase; (b) curves 1–4, before and after 3, 21, and 140 min of illumination, respectively, of 16.8 μM wild-type lipoamide dehydrogenase.

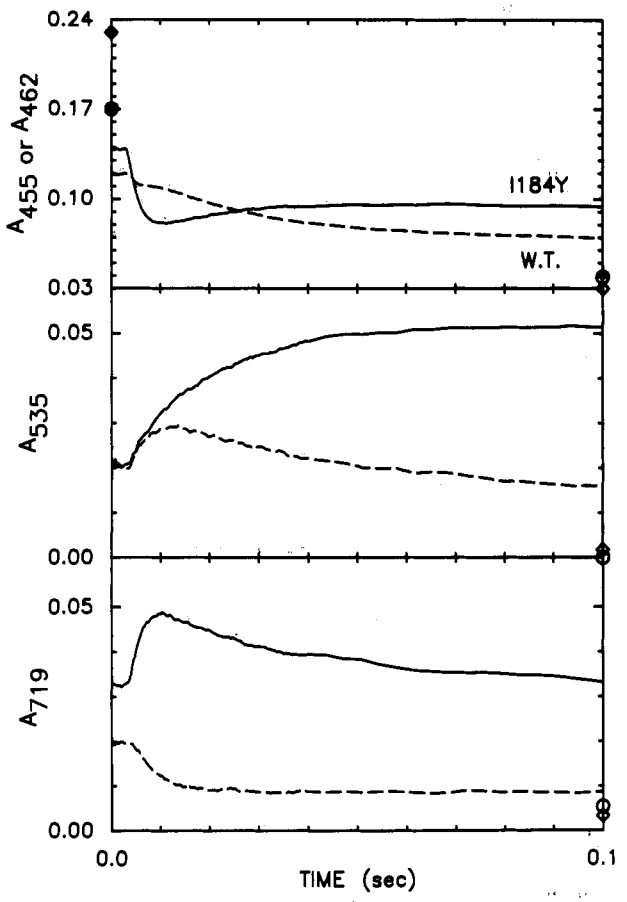


FIGURE 5: Reduction of I184Y and wild-type lipoamide dehydrogenases with NADH. Stopped-flow patterns are at pH 7.6 and 4 °C of the first 100 ms of the anaerobic reduction of 10.2 μM I184Y (—) and 7.5 μM wild-type (---) lipoamide dehydrogenases with 139 and 100 μM NADH, respectively. The symbols shown on the left-hand and right-hand vertical axes represent the initial and final (at 40 s) absorbance values, respectively, obtained for wild type (●, ○) and I184Y (◆, ◇).

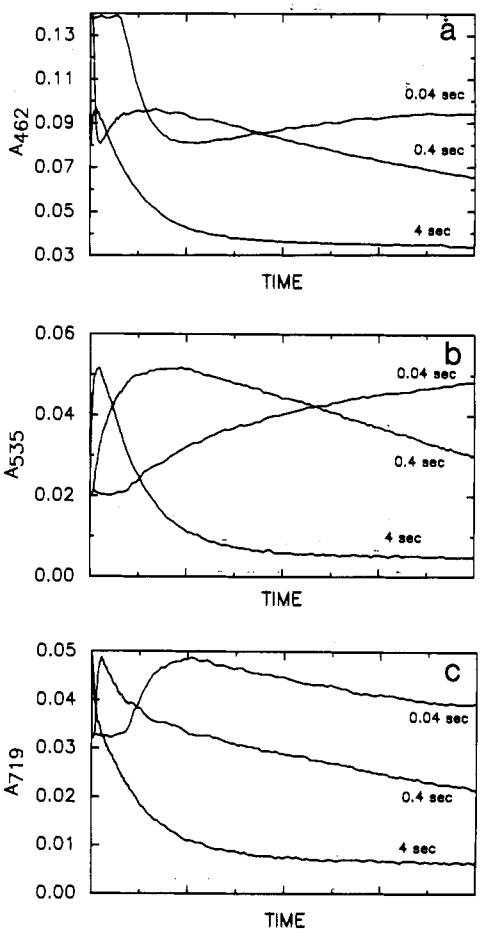


FIGURE 6: Kinetics of reduction of I184Y by NADH. Equal volumes of 20 μM I184Y and 183 μM NADH were mixed anaerobically and observed in the 2-cm light path cell of the stopped-flow spectrophotometer at 4 °C and pH 7.6. Trace at (a) 462 nm, (b) 535 nm, and (c) 719 nm. The traces shown are for the first 0.04, 0.4, and 4 s, respectively, as indicated on each figure.

complete in 10 ms, followed by reduction of the flavin (EH_4). By contrast, with I184Y, the last third of the FAD reduction is observed in the first 10 ms at 462 and 719 nm, and the transfer of electrons to the disulfide is seen in the next 90 ms at 535 nm.

The three phases of this reaction are shown for I184Y in Figure 6. The absorbance at 462 nm decreased from an initial value of 0.2 (FAD reduction) and increased at 719 nm ($FADH_2$ to NAD^+ charge-transfer complex formation) in the fast phase, which was complete in 10 ms. The passage of electrons from $FADH_2$ to the disulfide to form the thiolate to FAD charge-transfer complex is complete after 100 ms. Finally, the absorbance at 535 nm decreases slowly in the final phase (reduction to EH_4). These traces could be fitted with the same three exponentials independent of the wavelength measured and are given in Table I. Rate constants for the first and third phases showed hyperbolic dependence on the NADH concentration, while the rate of the second phase did not change with NADH concentration (Figure 7). The same three phases were observed at pH 8.5, and again the second phase was independent of NADH concentration and approximately 9-fold slower than at pH 7.6. At pH 6.2, three phases were again observed, but now the fast phase was complete in the dead time at all concentrations of NADH tested (0.1–1 mM). The remaining two phases were also faster than at pH 7.6.

Reduction of both the wild-type and I184Y enzymes with $Lip(SH)_2NH_2$ showed dead time formation of the typical EH_2 charge-transfer absorption with no absorbance at 719 nm, followed by a $Lip(SH)_2NH_2$ concentration-dependent conversion to the fully reduced EH_4 state. It was not possible to analyze the first phase of reduction of either enzyme, because the reaction was complete within the dead time of the

Table I: Comparison of Kinetic Parameters for the Reductive Half-Reactions of I184Y and Wild-Type Lipoamide Dehydrogenase by Substrates

	I184Y	wild type
	Reduction with NADH	
k_1 (s^{-1})	1000	>1000
$K_{d(1)}$ (μM)	90	ND[a]
k_2 (s^{-1})	50	very fast
k_3, k_4	in rapid equilibrium	in rapid equilibrium
k_5 (s^{-1})	2.8	100
$K_{d(5)}$ (μM)	30	50
	Reduction with $Lip(SH)_2NH_2$	
k_1', k_2'	very fast	very fast
$K_{d(1)}'$ (μM)	ND	~150
k_3', k_4'	in rapid equilibrium	in rapid equilibrium
k_5' (s^{-1})	0.17	0.60[b]
		0.35[c]
$K_{d(5)}'$ (μM)	~400–500	300[b]
		860[c]

[a] ND, not determined. [b] The rates and dissociation constants for the first and third phases were calculated as in Figure 6 and are interpreted in Scheme I. [c] Reduction of wild-type lipoamide dehydrogenase by $Lip(SH)_2NH_2$ from EH_2 to EH_4 was biphasic with different K_d values associated with each phase.

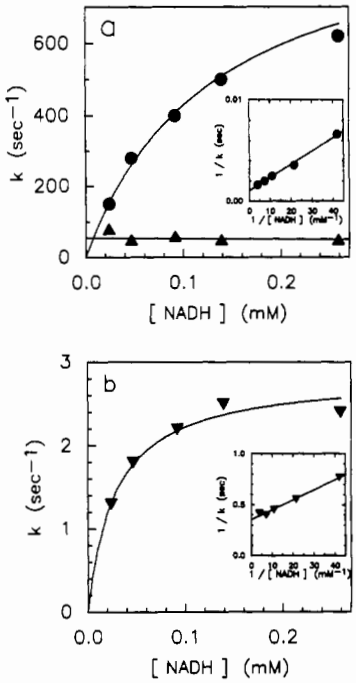


FIGURE 7: Effect of NADH concentration on the pseudo-first-order rate constant of the anaerobic reduction of I184Y: (a) rate of the first (●) and second (▲) phases (k_1 and k_2 as defined in Scheme I); (b) rate of the third (▼) phase (k_5 as defined in Scheme I). Data were from a series of experiments like those of Figure 5 with a range of NADH concentrations. Final NADH concentrations were 23.8, 46.6, 91.5, 139, and 258 μM. Insets: Double-reciprocal plots of k_1 and k_5 vs NADH concentration.

stopped-flow apparatus. This is in contrast to the pig heart enzyme where the reduction by $Lip(SH)_2NH_2$ does not proceed beyond the EH_2 stage and has a limiting rate constant of 555 s^{-1} at 25 °C; a slower phase is attributed to the unnatural substrate enantiomer (Massey et al., 1960; Matthews et al., 1977, 1979). The slower phase of the I184Y reduction (EH_2 to EH_4) could be fitted with a single exponential decay, but that of the wild-type enzyme required two exponentials with both phases having the same amplitude. The apparent first-order rate constants (k_5) all depended hyperbolically on the $Lip(SH)_2NH_2$ concentration.

Scheme I summarizes these results showing our interpretation of the pathways of reduction by NADH and $Lip(SH)_2NH_2$.

Discussion

It is clear from numerous studies [see Ghisla and Massey (1986) for a review] that the physical properties of flavoproteins and the chemical reactivity of the flavin chromophore are modulated by the protein environment. However, the specific effects brought about by specific residues interacting with the flavin are only now beginning to be understood. It is obvious that detailed studies of the effects of single-site mutations in flavoproteins will yield information of a nature applicable to flavoproteins in general, as well as specific information concerning the effects of such mutations on the individual enzymes. For this reason, we wish to document in this and subsequent papers those properties which may throw light on the understanding of the effect of the protein environment on the properties of the protein-bound flavin.

The replacement of Ile^{184} by a tyrosine residue in *E. coli* lipoamide dehydrogenase brings about dramatic changes in the spectral characteristics of the oxidized enzyme and in its catalytic properties. The absorbance is red shifted, the negative circular dichroism band in the visible wavelength region becomes positive, and there is nearly complete quenching of the FAD fluorescence. The altered enzyme, with respect to these properties at least, is more like glutathione reductase than wild-type lipoamide dehydrogenase. Since FAD is the only chromophore in the visible wavelength region, these spectral changes reflect the perturbation of the FAD environment by the single substitution of a tyrosine ring for an aliphatic side chain. These results provide strong support for the concept that the quenching of the flavin fluorescence commonly found with flavoproteins is due in large part to correct juxtaposition of the flavin to aromatic amino acid residues. The significance of these fluorescence changes is emphasized by the finding that wild-type lipoamide dehydrogenase of *Pseudomonas putida*, which has a tyrosine residue instead of isoleucine (Burns et al., 1988), also shows only 3% the fluorescence of free FAD.[2] However, an analogous mutation in *E. coli* glutathione reductase has been reported (Berry et al., 1989). In this study, Tyr^{177} (homologous with Tyr^{197} in the human enzyme) was changed to Phe (Y177F), Ser (Y177S), and Gly (Y177G). These alterations all resulted in 25-fold higher fluorescence than in wild-type glutathione reductase, although the fluorescence intensity of these mutants was still low, about 40% that of free FAD. The fact that Y177F had fluorescence suggests either that the phenolic side chain was more effective or that the Phe ring was less well positioned.

In reactions catalyzed by lipoamide dehydrogenase and glutathione reductase, each enzyme cycles between oxidized and two-electron-reduced forms in turnover. Thus, in the working hypothesis presented in Scheme I, physiological catalysis by lipoamide dehydrogenase is from E, counterclockwise through the EH_2 species, back to E, while catalysis of the NADH–$LipS_2NH_2$ reaction proceeds clockwise through the same species. Anaerobically, in the presence of excess $Lip(SH)_2NH_2$ or NADH, further reduction to EH_4 occurs.

I184Y lipoamide dehydrogenase has only ~9% the catalytic activity of the wild-type enzyme in the $Lip(SH)_2NH_2$–NAD^+ reaction, and there is profound inhibition by excess $Lip(SH)_2NH_2$ in the mutated enzyme. The activity of I184Y in the reverse reaction, NADH–$LipS_2NH_2$, is also sensitive to the NADH concentration. As catalysis involves shuttling of the enzyme between the oxidized and EH_2 species, the profound inhibition by both $Lip(SH)_2NH_2$ and NADH displayed by I184Y implies an increase in the EH_2/EH_4 redox potential, rendering the mutant enzyme more susceptible to reduction to the inactive EH_4 species. It is unlikely that increased binding affinity of NADH contributes to these results in light of the structure of glutathione reductase, which shows that binding is driven by interactions with the ADP-ribose moiety of NADPH and that Tyr^{197} is not near this binding region (Pai & Schulz, 1983; Karplus & Schulz, 1987). Determination of the E/EH_2 and EH_2/EH_4 redox potentials is rendered very difficult in the *E. coli* enzyme due to the multiple species contributing to EH_2 (Wilkinson & Williams, 1979) (cf. Scheme I).

The stopped-flow data indicate that reduction of the FAD of I184Y by NADH is almost as fast as with the wild-type enzyme but that, in the mutated enzyme, the rate of reduction of the disulfide by $FADH^-$ is much slower. Thus, in the first phase, the absorbance decrease at 460 nm, indicative of FAD reduction, and the absorbance increase at 719 nm, indicative of a charge-transfer transition from $FADH^-$ to NAD^+ (species II, Scheme I), are more than half complete in the dead time ($k_1 \simeq 1000\ s^{-1}$, Table I). In the second phase, the absorbance

[2] C. H. Williams, Jr., L. D. Arscott, and J. R. Sokatch, unpublished data.

increase at 535 nm, indicating formation of the thiolate to FAD charge-transfer transition (species IV, Scheme I), and the absorbance increase at 460 nm and decrease at 719 nm, indicating $FADH^-$ reoxidation, are complete in 100 ms (k_2 = 50 s^{-1}). The diminished rate of disulfide reduction by $FADH^-$ in I184Y may be due in part to the lower concentration of $FADH^-$ resulting from the increase in the flavin pK_a in the altered enzyme. The EH_2 charge-transfer complex (species IV, Scheme I) breaks down in the third phase as FAD is reduced by a second equivalent of NADH.

With reduction of the disulfide by $FADH^-$ occurring by way of a covalent adduct (species III, Scheme I) (Thorpe & Williams, 1976b), the rate of adduct breakdown must be fast, since no absorbance increase at 390 nm was observed, which would be indicative of accumulation of thiol C4a adduct. Species V–II must be in rapid equilibrium in the reverse direction, since the charge-transfer complex EH_2 (species IV, Scheme I) is formed in the dead time of reduction by Lip-$(SH)_2NH_2$ and since the subsequent slower reduction of this species to EH_4 is hyperbolically dependent on the Lip-$(SH)_2NH_2$ concentration.

Wild-type *E. coli* lipoamide dehydrogenase showed a similar stopped-flow pattern with NADH except that the absorbance increase at 719 nm was complete in the 3-ms dead time of the stopped-flow apparatus. The band at 719 nm seen in NADH reduction of I184Y and in reoxidation of the photoreduced enzyme in the presence of NAD^+ is most logically attributed to the charge-transfer interaction between reduced flavin and NAD^+ (Massey & Palmer, 1962; Williams, 1965). These similarities lead us to assume that the electron pathway and intermediate species are the same for the first two phases in I184Y and wild-type *E. coli* lipoamide dehydrogenases as those in pig heart lipoamide dehydrogenase.

Transient stabilization of the charge-transfer complex (species IV, Scheme I) upon reduction with NADH is observed with both I184Y and wild-type enzymes. More of species IV is actually observed in the 100-ms time frame with I184Y than with the wild-type enzyme, due to the much smaller rate of reduction to the EH_4 level (Table I). Both enzymes are further reduced to EH_4 even by APADH, which has a redox potential of −258 mV (Anderson & Kaplan, 1959), provided that the pH was less than 7.0. The spectral characteristics of EH_4 indicate that the N-1 proton of $FADH_2$ dissociates with a pK_a between 6.0 and 7.6 in the wild-type enzyme and between 7.6 and 8.5 in I184Y. Photoreduction of both enzymes to the EH_2 level is via a neutral semiquinone, and further reduction to EH_4 is facile. It should be noted that reduction of glutathione reductase from the EH_2 to the EH_4 state requires a strong reductant, implying a low $FAD/FADH_2$ redox potential. In contrast, the results with I184Y suggest a relatively high value for the potential of this couple. Air reoxidation of EH_4 is slower in I184Y than in the wild-type enzyme, supporting the notion that the side chain of tyrosine shields the flavin from oxygen (Pai & Schulz, 1983).

This study has demonstrated substantial effects of replacement of the isoleucine residue at position 184 with a tyrosine on the spectral properties of the enzyme-bound FAD as well as on its redox properties. These findings suggest strongly that the conclusions drawn from X-ray crystal structure determinations of glutathione reductase can be extended to the closely related enzyme lipoamide dehydrogenase. Glutathione reductase has a tyrosine residue at the homologous position, and the X-ray crystallographic data show a displacement of the tyrosine ring in the presence of $NADP^+$ (Thieme et al., 1981). In lipoamide dehydrogenase the introduction of a tyrosine residue at the equivalent position clearly alters the interaction of NAD^+ with the enzyme flavin, consistent with the aromatic ring having to be displaced somewhat in order for the pyridine nucleotide to gain access to the flavin, as with glutathione reductase. In addition, this alteration markedly slows the rate of electron transfer from $FADH_2$ to the redox-active disulfide, allowing the EH_2 species with the reduced flavin to be observed. Thus, in I184Y, the rate of one step in the reaction sequence has been altered, allowing us to observe and characterize a previously hypothetical intermediate. As expected from the perceived structure of the active site, the reaction with $Lip(SH)_2NH_2$ is not significantly affected by the mutation because it occurs on the opposite (*si*) side of the flavin from the site of alteration.

In summary, the replacement in *E. coli* lipoamide dehydrogenase of Ile^{184} by a tyrosine residue conveys to the enzyme many of the same properties exhibited by human erythrocyte glutathione reductase. At the same time the results demonstrate the exquisite sensitivity of the physical and catalytic properties of the flavin chromophore to its protein environment.

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Registry No. Ile, 73-32-5; Tyr, 60-18-4; NAD, 53-84-9; NADH, 58-68-4; FAD, 146-14-5; $Lip(SH)_2NH_2$, 3884-47-7; glutathione reductase, 9001-48-3; lipoamide dehydrogenase, 9001-18-7.

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Functional Reconstitution of the γ-Aminobutyric Acid Transporter from Synaptic Vesicles Using Artificial Ion Gradients†

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ABSTRACT: The γ-aminobutyric acid transporter of rat brain synaptic vesicles was reconstituted in proteoliposomes, and its activity was studied in response to artificially created membrane potentials or proton gradients. Changes of the membrane potential were monitored using the dyes oxonol VI and 3,3′-diisopropylthiodicarbocyanine iodide, and changes of the H^+ gradient were followed using acridine orange. An inside positive membrane potential was generated by the creation of an inwardly directed K^+ gradient and the subsequent addition of valinomycin. Under these conditions, valinomycin evoked uptake of [^{3}H]GABA which was saturable. Similarly, [^{3}H]glutamate uptake was stimulated by valinomycin, indicating that both transporters can be driven by the membrane potential. Proton gradients were generated by the incubation of K^+-loaded proteoliposomes in a buffer free of K^+ or Na^+ ions and the subsequent addition of nigericin. Proton gradients were also generated via the endogenous H^+ ATPase by incubation of K^+-loaded proteoliposomes in equimolar K^+ buffer in the presence of valinomycin. These proton gradients evoked nonspecific, nonsaturable uptake of GABA and β-alanine but not of glycine in proteoliposomes as well as protein-free liposomes. Therefore, transporter activity was monitored using glycine as an alternative substrate. Proton gradients generated by both methods elicited saturable glycine uptake in proteoliposomes. Together, our data confirm that the vesicular GABA transporter can be energized by both the membrane potential and the pH gradient and show that transport can be achieved by artificial gradients independently of the endogenous proton ATPase.

Signals are conducted between neurons via the release of small molecules, the neurotransmitters. It is generally accepted that nonpeptide neurotransmitters are stored in high amounts in nerve terminals where they are concentrated in synaptic vesicles. Upon stimulation, synaptic vesicles exocytose and release their content into the synaptic cleft. The synaptic vesicle membrane is then retrieved from the plasma membrane by endocytosis to reform synaptic vesicles. These vesicles are reloaded with neurotransmitter and are reset to undergo another round of exo–endocytosis.

It is well established that synaptic vesicles possess the capacity to sequester and store neurotransmitters. High concentrations of acetylcholine (Whittaker et al., 1964), monoamines [reviewed by Philippu and Matthaei (1988)], glutamate (Burger et al., 1989), GABA,[1] and glycine (Burger et

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