Modulation of the Oxidation–Reduction Potential of the Flavin in Lipoamide Dehydrogenase from *Escherichia coli* by Alteration of a Nearby Charged Residue, K53R†

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ABSTRACT: The ε-amino group of a lysine residue occupies a position within bonding distance of the flavin N5 and the bound NADPH pyridinium C4′ in glutathione reductase, and it has been suggested that this positive charge influences the redox potential of the FAD [Pai & Schulz (1983) *J. Biol. Chem. 258*, 1752]. A conserved lysine residue occupies a similar position in lipoamide dehydrogenase. This residue has been replaced by an arginine in lipoamide dehydrogenase from *Escherichia coli* to give K53R. The spectral and redox properties of the FAD in K53R as well as the interaction of the flavin with bound NAD^+ are profoundly affected by the change. K53R does not catalyze either the dihydrolipoamide–NAD^+ or the NADH–lipoamide reactions except at very low concentrations of the reducing substrate. The absorbance spectrum of K53R in the visible and near-ultraviolet is little changed from that of wild-type enzyme, but in contrast, the spectrum of K53R is sensitive to pH with an apparent pK_a = 7.0. Unlike the wild-type enzyme, the binding of β-NAD^+ to K53R alters the spectrum and indicates an apparent K_d = 7.0 μM at pH 7.6. The flavin fluorescence is partially quenched, and the visible and near-ultraviolet circular dichroism spectrum is changed by β-NAD^+. K53R is extensively reduced (mostly EH_4) by 2 equiv of dihydrolipoamide/FAD while the wild-type enzyme is only partially reduced (mostly EH_2). The rate of this reduction is lowered by approximately 3-fold relative to the wild-type enzyme. Reduction of K53R by NADH at pH 8.5 results in the rapid appearance of a broad band at 750 nm interpreted as an $FADH_2$–NAD^+ charge-transfer complex. The rate of the reduction is about half that observed with the wild-type enzyme. Neutral flavin semiquinone appears during photoreduction of K53R. Addition of β-NAD^+ to the photoreduced enzyme results in the immediate appearance of the $FADH_2$–NAD^+ charge-transfer complex. Sensitivity of K53R to four-electron reduction indicates that the redox potential of the FAD has been raised relative to that in the wild-type enzyme, and this was confirmed by direct measurement of the redox potential.

Lipoamide dehydrogenase is a member of the family of FAD-containing pyridine nucleotide-disulfide oxidoreductases (Massey et al., 1960), and catalyzes the reversible reaction:

$$Lip(SH)_2NH_2^1 + \beta\text{-}NAD^+ \rightleftarrows LipS_2NH_2 + \beta\text{-}NADH + H^+ \quad (1)$$

The X-ray crystal structures of *Azotobacter vinelandii* lipoamide dehydrogenase at 2.2-Å resolution and of human erythrocyte glutathione reductase at 1.54 Å are very similar. They show that the pyridine nucleotide binding site is on the *re* side while the redox-active disulfide and the dithiol substrate binding site are on the *si* side of the isoalloxazine of FAD (Thieme et al., 1981; Karplus & Schulz, 1987; Mattevi et al., 1991; Schierbeek et al., 1989). The ammonium function of Lys^{66} is near the N5 of the isoalloxazine ring and the C4′ of the bound pyridinium ring of NADPH in glutathione reductase, and Lys^{57} occupies a similar position in *A. vinelandii* lipoamide dehydrogenase. The lysine residue forms an ion pair with a glutamate residue in both proteins with the positive end of the salt bridge nearer the flavin (Karplus & Schulz, 1987; Mattevi et al., 1991). It has been suggested that the positive charge affects the FAD electrostatically (Pai & Schulz, 1983). Lys^{53} and Glu^{188} occupy the homologous positions in lipoamide dehydrogenase of *Escherichia coli* (Stephens et al., 1983).

Residues in the pyridine nucleotide binding site of lipoamide dehydrogenase from *E. coli*, including Lys^{53} and Glu^{188}, have been modified by site-directed mutagenesis in order to study their influence on the properties of the flavin and on the interaction of pyridine nucleotide and flavin (Allison et al., 1988; Williams et al., 1989; Russell et al., 1989). Substituting

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[1] Abbreviations: K53R or I184Y, site-directed mutation of *E. coli* lipoamide dehydrogenase with lysine-53 replaced with an arginine residue or isoleucine-184 replaced with a tyrosine residue, respectively; $Lip(SH)_2NH_2$, 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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Ile[184] by tyrosine leads to profound effects on the enzymatic and spectral properties (Maeda-Yorita et al., 1991). Ile[184] is homologous with a tyrosine residue in the pyridine nucleotide binding site of glutathione reductase (Stephens et al., 1983; Krauth-Siegel et al., 1982). This modification (I184Y) caused the visible absorbance, fluorescence, and circular dichroism of lipoamide dehydrogenase to mimic glutathione reductase. The relative rates of the steps in reduction by NADH are also altered (Maeda-Yorita et al., 1991).

The wide variations in the properties of the flavin in each flavoprotein are imposed by its specific surroundings (Ghisla & Massey, 1986). Here we report the effects of replacing Lys[53] by arginine (K53R). Although this could be considered a minimal perturbation, the effects on the properties of the enzyme are large. As predicted, the change in the exact position and the charge density distribution in an arginine residue relative to a lysine residue alters the spectral properties and redox potential of the flavin as well as the interaction of the flavin with bound NAD^+.

EXPERIMENTAL PROCEDURES

Growth of *E. coli*, purification of wild-type lipoamide dehydrogenase and K53R, anaerobiosis, spectral measurements, anaerobic techniques, and sources of reagents were the same as those used previously (Maeda-Yorita et al., 1991). The concentration of enzyme samples was determined using an extinction coefficient of 11.3 mM^{-1} cm^{-1} at 455 nm for the enzyme-bound FAD in K53R and wild-type lipoamide dehydrogenase.

Site-directed mutagenesis of *E. coli* lipoamide dehydrogenase to generate K53R was carried out as described previously (Allison et al., 1988; Williams et al., 1989; Russell et al., 1989). Because the host *E. coli* strain (JRG1342 Δ*ace-lpd*) lacks the lipoamide dehydrogenase gene, there was no native enzyme present unless the cells were transformed with plasmid containing the wild-type gene.

Rapid reaction kinetics were measured with a stopped-flow spectrophotometer, and reaction kinetic data were analyzed using Program A written by C.-J. Chiu and D. P. Ballou, Department of Biological Chemistry, University of Michigan.

Redox Potentials in K53R. The enzyme in 0.1 M sodium/potassium phosphate buffer, pH 7.0, was mixed with phenosafranine, a dye of known redox potential (−252 mV), at 25 °C. The low-potential xanthine/xanthine oxidase system was used as electron donor (Massey, 1991). The input of electron equivalents into the enzyme was estimated by the increase in absorbance at 290 nm. This is an isosbestic point in the reduction of phenosafranine under the same experimental conditions, and the change in the flavin spectrum upon reduction is also small at this wavelength. Hence, the absorbance increase at this wavelength is due mainly to the conversion of xanthine to urate and can be used to estimate the number of reducing equivalents delivered to K53R by the xanthine/xanthine oxidase reducing system using an extinction change, $\Delta\epsilon = 9.92$ mM^{-1} cm^{-1}. Errors inherent in this system derive primarily from the fact that the isosbestic point occurs at a point in the phenosafranine spectra where the absorbance change is large. Because the reaction curves calculated for the flavin and the disulfide in Figure 10 do not display ideal Nernstian behavior, that is, the slopes are somewhat greater than would be predicted for a two-electron change, the estimated redox potentials are approximate. The reaction curve for the disulfide yields a potential equal to that calculated from independent experiments with the wild-type enzyme (Wilkinson & Williams, 1979). It should be noted that only qualitative conclusions have been based on these data, namely, that the three systems, phenosafranine, flavin, and disulfide, are reduced at similar potentials (see Results).

Spectra of the system were taken at 2-min intervals throughout the reduction with a Hewlett-Packard diode array spectrophotometer. The concentrations of the oxidized and reduced dye were calculated from the spectra of 540 nm after correcting for small amounts of neutral flavin semiquinone. These data were used to define the system potential. Percentage reduction of the enzyme flavin was estimated by the decrease in absorbance at 454 nm corrected for the contribution of phenosafranine to the absorbance, using an extinction coefficient of 8900 M^{-1} cm^{-1} for the difference between oxidized and reduced flavin in K53R. The concentration of reduced disulfide was calculated from the difference between the total electron equivalents introduced into the enzyme (determined at 290 nm) and those taken up by the flavin (determined at 454 nm).

RESULTS

Enzymatic Activity of K53R. K53R did not catalyze either the $Lip(SH)_2 + NH_2 \sim \beta\text{-}NAD^+$ or the $\beta\text{-}NADH \sim LipS_2 + NH_2$ reaction effectively. The specific activity of the $Lip(SH)_2 + NH_2 \sim \beta\text{-}NAD^+$ reaction, at pH 7.6 and 25 °C with 200 μM DL-$Lip(SH)_2 + NH_2$ and 1 mM $\beta\text{-}NAD^+$, was 27 mol min^{-1} (mol of FAD)$^{-1}$, and that of the $\beta\text{-}NADH \sim LipS_2 + NH_2$ reaction, at pH 7.6 and 25 °C with 80 μM β-NADH, 1.3 mM DL-$LipS_2 + NH_2$, and 1 mM $\beta\text{-}NAD^+$, was 22 mol min^{-1} (mol of FAD)$^{-1}$. These values were 0.1% and 0.2% of those of wild-type lipoamide dehydrogenase from *E. coli* under the same conditions, respectively (Meada-Yorita et al., 1991; Sahlman & Williams, 1989). The pH dependence of the two activities was the same for K53R as those of wild-type enzyme and the optimum pH around 7.6.

Apparent strong inhibition by the electron donor in both reactions and strong dependence on the concentration of $\beta\text{-}NAD^+$ in the latter reaction were observed with K53R. This precluded the determination of the usual kinetic parameters, V_{max} and K_m. The dependence of the rate of the $\beta\text{-}NADH \sim LipS_2 + NH_2$ reaction on the concentration of $\beta\text{-}NAD^+$ was maximal for K53R at 0.2 mM but at 1 mM for wild-type enzyme (Wilkinson & Williams, 1981). The rate of β-NADH consumption by K53R monitored at 340 nm depended on the concentration of the substrates. There was a lag phase in β-NADH consumption which became longer with higher concentrations of β-NADH, or with lower concentrations of DL-$LipS_2 + NH_2$, consistent with the susceptibility of the enzyme to inhibition by excess reducing substrate.

Spectral Properties of K53R in the Oxidized State. While wild-type lipoamide dehydrogenase shows the same absorption spectrum in the visible wavelength region over a wide pH range (Wilkinson & Williams, 1979), that of K53R varied. The peak position of the first absorption band (first π–π^* transition of FAD) of K53R shifted from 452 nm at pH 8.5 to 456 nm at pH 5.0 without a significant change in intensity or fine structure of the FAD absorbance (Figure 1). A pK_a of 7 was determined from the difference spectra (Figure 1, inset). However, the second absorption band around 365 nm (second π–π^* transition band of FAD) of K53R did not show any pH-dependent shift of peak position. The relative intensity of the second absorption peak to the first of K53R at pH 7.6 was 0.79, slightly higher than 0.72 of wild-type enzyme.

A very substantial change in the FAD absorbance of K53R was induced by $\beta\text{-}NAD^+$ (Figure 2). In the presence of excess $\beta\text{-}NAD^+$, both the first and second absorption bands were

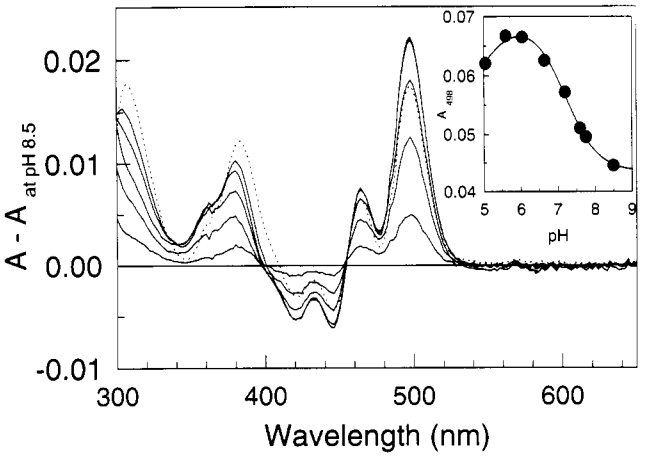


FIGURE 1: Effect of pH on the absorption spectrum of K53R. The pH of 12.7 μM K53R at 4 °C in 0.1 M potassium phosphate buffer, pH 7.6, was changed to 8.5, 7.7, 7.2, 6.65, 6.05, 5.6, and 5.0 by adding powdered $KHCO_3$, K_2CO_3, or citric acid. The peak was shifted from 452 nm at pH 8.5 to 456 nm at pH 5.0 without an intensity change. Difference spectra were generated by subtracting the spectrum at each pH from the spectrum at pH 8.5. The differences became larger as the pH is decreased to 5.6. The protein showed signs of changes at pH 5 (dotted). Inset: the difference at 498 nm as a function of pH indicates a pK_a value of 7.

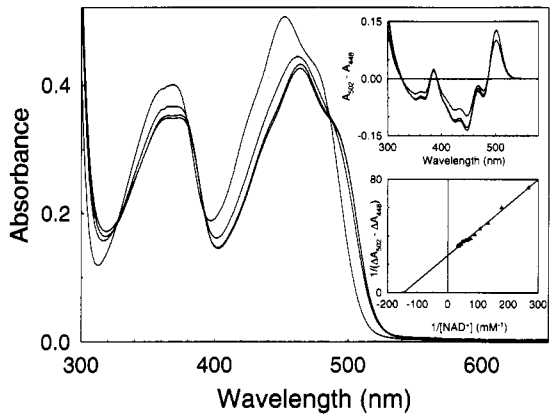


FIGURE 2: Effect of β-NAD$^+$ on the absorption spectrum of oxidized K53R. K53R (224 μM in 0.1 M potassium phosphate buffer, pH 7.6, at 4 °C) was titrated with β-NAD$^+$. The final ratios of β-NAD$^+$ to FAD were 0, 0.825, 2.06, and 3.26. The absorbance decreased and red-shifted as the ratio increased. No pH change was observed after addition of β-NAD$^+$. Spectra were measured in a 2-mm light path cell. Difference spectra are shown in the top inset: the spectrum at each level of β-NAD$^+$ was subtracted from the spectrum of K53R. The bottom inset shows a reciprocal plot of data from the titration of 4.98 μM K53R with β-NAD$^+$ in a 10-mm light path cell at pH 7.6 and 4 °C.

red-shifted to 465 and 370 nm, respectively, and the intensity at the absorption maxima was reduced by 15%. The fine structure of each absorption band also changed. The spectra with β-NAD$^+$ present were the same at pH 6.0, 7.6, and 8.5. The dissociation constants for β-NAD$^+$ at 4 °C were 7 and 80–100 μM at pH 7.6 and 6.0, respectively. Wild-type enzyme did not show any change on addition of β-NAD$^+$. Although a 2-nm red-shift in the peak position in the first absorption band was observed with the same concentration of β-AAD$^+$ or β-APAD$^+$ as with β-NAD$^+$, neither β-NADP$^+$ nor α-NAD$^+$ induced any change in the visible absorption of K53R.

The fluorescence excitation spectrum of FAD in K53R was essentially the same as that of the absorption spectrum with peaks at 365 and 454 nm (Figure 3). The emission spectrum had a peak at 520 nm when excited at 450 nm. This peak position was identical to that of wild-type lipoamide dehydrogenase, and was blue-shifted by 5 nm from that of free

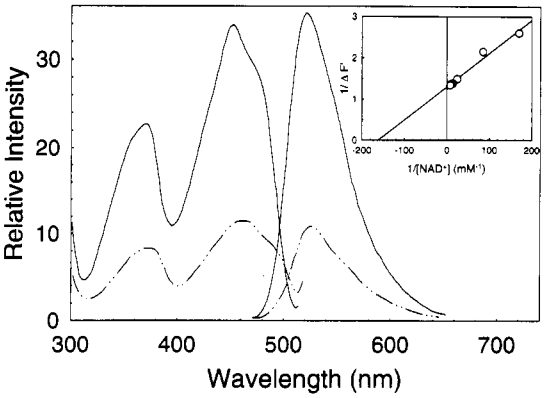


FIGURE 3: Fluorescence excitation and emission spectra of K53R. Enzyme (4.98 μM) in the absence (—) and presence (– ·· –) of 190 μM β-NAD$^+$ at pH 7.6 and 4 °C. Excitation spectra were monitored with an emission wavelength of 525 nm, and emission spectra were measured with an excitation wavelength of 450 nm. The inset shows a Benesi–Hildebrand plot of the titration of 5.13 μM K53R at pH 7.6 and 4 °C with β-NAD$^+$ monitoring the fluorescence intensity: $\Delta F = F_0 - F'$, where F_0 is the fluorescence intensity without β-NAD$^+$ and F' is the fluorescence intensity with β-NAD$^+$. Correction has been made for the volume change.

FAD. The fluorescence intensity at 525 nm of K53R at pH 7.6 and 4 °C was 44% of that of wild-type enzyme, but it was still higher than that of pure FAD by 2.5-fold. In contrast to wild-type enzyme, K53R fluorescence intensity showed a remarkable dependence on pH, approximately doubling as the pH increased, with a pK_a value of 7. This value was identical to that derived from the absorption difference spectra. Neither the excitation nor the emission peak positions were sensitive to pH.

β-NAD$^+$ quenched the FAD fluorescence of K53R to 33% of its original value at pH 7.6 (Figure 3). The peaks of the excitation spectrum were shifted to 372 and 462 nm, respectively, and the spectral shape in the presence of β-NAD$^+$ was altered. The peak of the emission spectrum in the presence of β-NAD$^+$ was also red-shifted to 525 nm. These observations indicated that at least two species were contributing to the fluorescence emission in the presence of β-NAD$^+$: a highly fluorescent β-NAD$^+$-free and a β-NAD$^+$-bound enzyme species of lower fluorescence. Fluorescence quenching indicated a binding constant for β-NAD$^+$ of 6 μM, the same as that measured by absorbance. The β-NAD$^+$-quenched fluorescence intensity was insensitive to pH above pH 5. Neither β-NADP$^+$ nor KCl quenched the FAD fluorescence.

The circular dichroism spectra of K53R in the near-UV and UV wavelength regions were the same as those of wild-type enzyme. The CD spectrum of K53R in the visible wavelength region (Figure 4) was also similar to that of wild-type enzyme except for a slight blue-shift in the peak position. Because the FAD molecule is asymmetric and no other chromophore is present in lipoamide dehydrogenase, the CD spectrum in the visible wavelength region is considered to be due to the interaction of the isoalloxazine part with the ribityl side chain of FAD and with the protein (Edmondson & Tollin, 1971). Therefore, it may be concluded that no significant change of the stereochemistry of isoalloxazine was induced in K53R.

Addition of β-NAD$^+$ induced changes in the CD spectrum of K53R (Figure 4). The intensity of the negative Cotton effect around 450 nm was decreased, and the positive Cotton effect around 370 nm was red-shifted to 375 nm, and its intensity was increased 1.2-fold. Because β-NAD$^+$ does not have an absorption band in the visible wavelength region, this spectral perturbation was likely due to a change in the

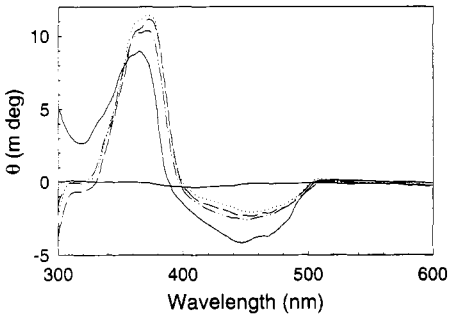


FIGURE 4: Circular dichroism spectra of K53R in the visible wavelength region and the effect of β-NAD^+. The samples were the same as in Figure 2, and the same 2-mm light path cell was used: (—) 0; (– · · –) 0.83; (– –) 2.06; (···) 3.26 equiv of β-NAD^+.

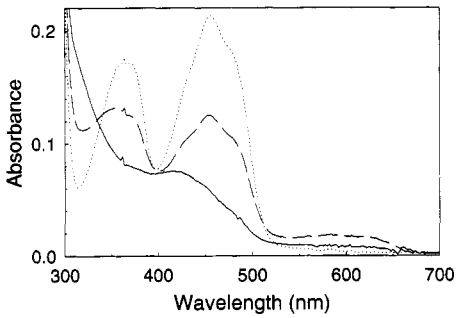


FIGURE 5: Anaerobic reduction of K53R with DL-dihydrolipoamide. (···) 13.6 μM K53R; (– –) after addition of 1.1 equiv of DL-Lip-$(SH)_2NH_2$; (—) with 2.2 equiv of DL-Lip$(SH)_2NH_2$, all at pH 7.6 and 4 °C.

environment of the isoalloxazine ring by direct interaction with β-NAD^+. A small positive Cotton effect at 330 nm changed sign upon the addition of β-NAD^+, indicating a possible conformational change in the protein moiety, including possibly the redox-active disulfide bond.

Reduction of K53R with DL-*Lip$(SH)_2NH_2$*. Figure 5 shows the spectra of oxidized K53R and enzyme reduced with 1.1 and 2.2 equiv of DL-Lip$(SH)_2NH_2$ per enzyme-bound FAD. The L-stereoisomer is active, but in the relatively slow observation mode used here, all of the added reductant is effective (Wilkinson & Williams, 1979). The broad band centered at 600 nm, prominent after addition of the first equivalent, is typical of the neutral semiquinone of FAD. This is in contrast to the observation with wild-type enzyme in titration with DL-Lip$(SH)_2NH_2$, where all of the long-wavelength absorbance is due to the thiolate–flavin charge-transfer species (Williams, 1965). The absence of a marked blue-shift in the band at 454 nm also indicates that no charge-transfer species has formed. The failure to observe charge transfer could be due to poor positioning of the donor and acceptor, or it could be due to a change in the relative oxidation–reduction potentials of the disulfide and the FAD. The quantity of semiquinone is small, indicating extensive disproportionation. The second equivalent of DL-Lip-$(SH)_2NH_2$ leads to almost complete reduction, and the neutral dihydroflavin ($FADH_2$) with an absorption shoulder at 420 nm is the product. The same species is observed in wild-type enzyme, but the extent of reduction with this amount of dihydrolipoamide is far greater with K53R, again suggesting an increase in the redox potential of the FAD in the mutant enzyme.

Kinetics of the Reduction of K53R with DL-*Lip$(SH)_2NH_2$*. Reduction of wild-type lipoamide dehydrogenase with excess dihydrolipoamide leads to the formation of the thiolate–flavin charge-transfer complex in the dead time of about 3 ms. The

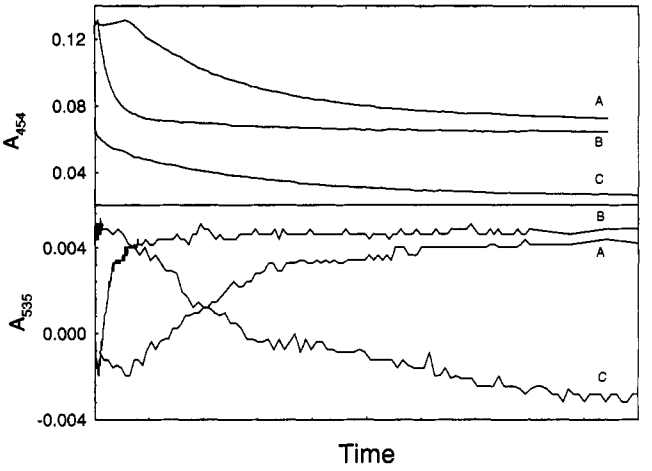
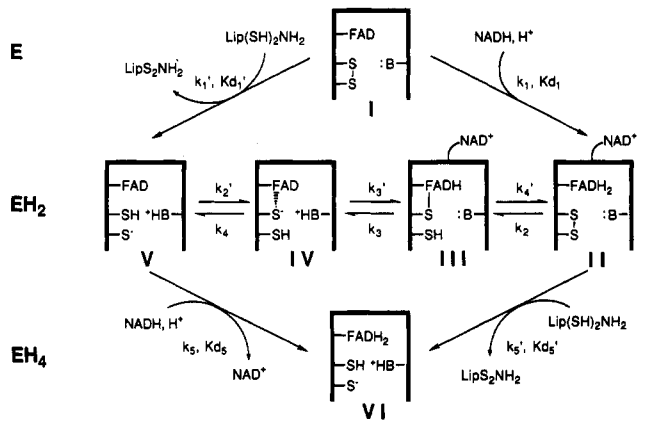


FIGURE 6: Kinetics of the reduction of K53R with DL-Lip$(SH)_2NH_2$. K53R (15.3 μM) was mixed under anaerobic conditions at pH 7.6 and 4 °C with 2.08 equiv of DL-Lip$(SH)_2NH_2$ at a 1:1 volume ratio. The light path was 2 cm. Time scales are (A) 0–50 ms, (B) 0–500 ms, and (C) 0–300 s.

Scheme 1



extinction at 530 nm is the same as that observed with the pig heart enzyme (Williams, 1976). However, whereas the charge-transfer complex with excess DL-Lip$(SH)_2NH_2$ is stable with time in the latter enzyme, the 530-nm absorbance of the *E. coli* enzyme disappears due to the transfer of electrons from the dithiol to the FAD and to further reduction—to EH_4 (Wilkinson & Williams, 1979). Some charge-transfer complex is formed rapidly when K53R is reduced with DL-Lip-$(SH)_2NH_2$ as seen in Figure 6. (About one-third of the reaction is missed in the mixing time of the instrument). The charge transfer observed is approximately 20% that seen with wild-type enzyme.

The reaction kinetics shown in Figure 6 are from an experiment in which reduction was effected with only 2 equiv of DL-Lip$(SH)_2NH_2$. In the rapid phase, extending to about 50 ms, only the L-stereoisomer reacts, and overreduction is slight (Wilkinson & Williams, 1979). The lower amount of charge-transfer complex (535 nm) and the lower extinction at 454 nm indicate that the equilibrium between the charge-transfer complex (Scheme 1, species IV) and the flavin-reduced species (Scheme 1, species II) favors the latter in K53R relative to wild-type lipoamide dehydrogenase. This again points to a change in the relative redox potentials of the disulfide and FAD, and we would argue that, since the altered residue in K53R is remote from the disulfide, the potential of the FAD has probably been raised by the more diffuse positive charge in the guanidinium group. As expected, the rate of the first phase was dependent on the concentration of dihydrolipoamide,

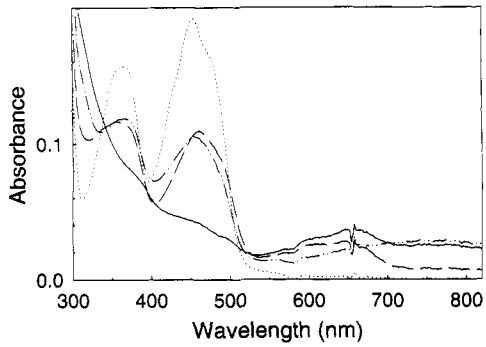


FIGURE 7: Reduction of K53R with β-NADH. K53R (15.2 μM in 0.1 M phosphate buffer, pH 7.6 and 4 °C) was reduced anaerobically with β-NADH. (···) Before addition; (– ·· –) 2.5 min after addition of 1.0 equiv of β-NADH; (– –) 150 min after addition of 1.0 equiv of β-NADH; (—) 125 min after addition of a second equivalent of β-NADH.

with a limiting rate of 310 s^{-1}, i.e., approximately one-third of the rate with wild-type enzyme, and with a $K_{d(app)}$ of 70 μM (results not shown).

The much slower further reduction of K53R (Figure 6) takes place at a rate attributed either to reduction of L-lipoamide by D-Lip(SH)$_2$NH$_2$ or to reduction of the enzyme by D-Lip(SH)$_2$NH$_2$ (Wilkinson & Williams, 1979). In this phase, the 530-nm absorbance disappears, and the 454-nm absorption falls to the level associated with fully reduced enzyme (EH_4). The slower phase, associated with conversion to EH_4, occurs at a rate of approximately 0.5 s^{-1} and is dependent on the reductant concentration.

Reduction of K53R with β-NADH and Its Analogs. Reduction of K53R with 1.0 equiv of β-NADH is shown in Figure 7. The spectrum 2 min after mixing showed the 454-nm band diminished by half and a broad peak centered at 750 nm—the reduced flavin–β-NAD^+ charge-transfer complex. There was no indication of the thiolate–flavin charge-transfer complex (absorbance at 530 nm and blue-shift in the 454-nm band) and very little, if any, semiquinone. In contrast, wild-type enzyme shows strong thiolate–flavin charge-transfer absorption and no evidence of reduced flavin–β-NAD^+ charge-transfer absorption (Williams, 1965). This indicates that in K53R, there is an equilibrium between species V and species II (Scheme 1) with both being present in equal concentrations. Subsequently, the FAD absorbance increased slightly and was red-shifted to 460 nm; the 750-nm band was replaced by a band at 620 nm, typical of the neutral flavin semiquinone. Isosbestic points at 304, 332, 352, 452, and 672 nm characterized these changes.

A second equivalent of β-NADH resulted in almost complete bleaching at 455 nm. Some semiquinone remained, but the amount was difficult to estimate due to the return of the 750-nm band (Figure 7). The formation of this latter band with only 1 or 2 equiv of β-NADH emphasizes the tight binding of β-NAD. During reoxidation by air, the peak associated with semiquinone disappeared first, as approximately 50% of the absorbance at 460 nm returned. The remaining flavin absorbance was regained as the 750-nm band disappeared. The final spectrum was again that expected for the oxidized K53R·β-NAD^+ complex (Figure 2).

Neither β-NADPH, β-APADH, nor α-NAD(H) induced the same types of spectral change as did β-NADH upon reduction of K53R, but spectra with these analogs were similar to those seen with DL-Lip(SH)$_2$NH$_2$; i.e., there was no indication of reduced flavin–oxidized pyridine nucleotide charge-transfer transitions. Compared to wild-type enzyme, the FAD in K53R was more easily reduced by β-APADH,

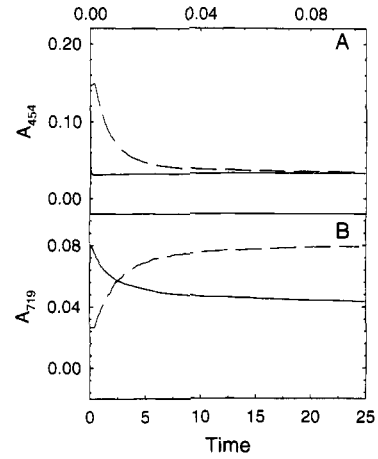


FIGURE 8: Kinetics of the reduction of K53R with β-NADH. K53R (17.9 μM) was mixed under anaerobic conditions at pH 7.6 and 4 °C with 191 equiv of β-NADH at a 1:1 volume ratio. The light path was 2 cm. (– –) 0–0.1 s, full scale; (—) 0–25 s, full scale.

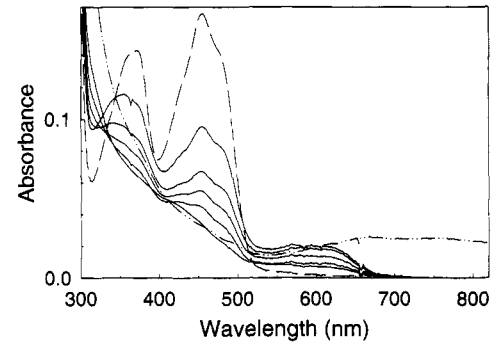


FIGURE 9: Anaerobic photoreduction of K53R. The enzyme was 15.1 μM at pH 7.6 and 4 °C with 1 mM EDTA and 1 μM deazaflavin. Photoreduction was for 0 (– –) and for 0.5, 1, 2, 4, and 8 min (solid curves, top to bottom), with a final addition of β-NAD^+ (– ·· –). Each spectrum was taken 20 min after photoirradiation.

which has midpoint potential higher by 60 mV (Kaplan, 1960) than that of β-NADH, again indicating a change in the redox potential of flavin as a result of the lysine to arginine substitution.

Kinetics of the Reduction of K53R with β-NADH. When K53R was mixed with 1.9 equiv of β-NADH, the spectral changes took place in two stages (Figure 8). In the first, the absorbance at 454 nm decreased, and that at 719 nm increased biphasically. The faster of the two rate constants depended hyperbolically on the concentration of β-NADH. The limiting rate was 540 s^{-1}, and the $K_{d(app)}$ was 74 μM. The changes at 535 and 620 nm were very small. The rate of the slower reaction did not depend on the pyridine nucleotide concentration. In the second stage, the flavin band appeared to rise slightly, and some of the 750-nm band disappeared, suggesting that β-NAD^+ may not bind as well to species VI as it does to species II (Scheme 1). When higher ratios of β-NADH to enzyme were used, the change at 454 nm in the slow phase was somewhat larger, and that at 719 nm was much larger. This suggests that β-NADH can bind to species VI (and perhaps can displace NAD^+ in species II, Scheme 1). The dependence of the rate of this process on β-NADH could not be determined.

Photoreduction. When K53R was photoreduced for 30 s (Figure 9) with EDTA as photoreductant and 5-deazaflavin as catalyst (Massey & Hemmerich, 1978), the resulting spectrum was very similar to that seen with 1 equiv of DL-Lip(SH)$_2$NH$_2$, but the amount of semiquinone was somewhat higher (cf. Figure 5). The absorption band of FAD at 454

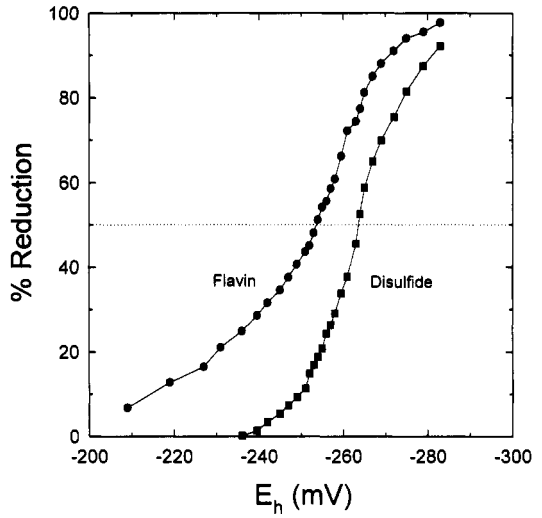


FIGURE 10: Reduction of K53R in the presence of phenosafranine. Enzyme (16 μM) in 0.1 M phosphate buffer, pH 7.0, 25 °C, in the presence of 20 μM phenosafranine, 200 μM xanthine, and 2 μM benzyl viologen was made anaerobic and reduction initiated by mixing, from a side arm, a final concentration of 50 nM xanthine oxidase. Spectra were recorded every 2 min, and the percent reduction of the enzyme flavin and the active-site disulfide was estimated as described under Experimental Procedures.

nm did not change shape or peak position. This indicated that there was no stable thiolate–flavin charge-transfer species. As expected, no absorption band around 750 nm was observed. It should be noted that immediately after photoirradiation, the amount of flavin semiquinone produced initially was larger than that shown after 20-min dark-equilibration in Figure 9. This indicates only partial thermodynamic stabilization of the semiquinone. Longer irradiation resulted in full flavin reduction with progressive loss of the band at 550–620 nm. Addition of β-NAD^+ induced slow formation of the long-wavelength absorption band around 750 nm, typical of reduced flavin–NAD^+ charge-transfer complexes (Massey & Palmer, 1962). This experiment emphasized an important difference between the wild-type and K53R enzymes. Under similar conditions, there is little absorbance around 750 nm with the wild-type enzyme, indicating a stronger interaction between β-NAD^+ and reduced flavin in K53R.

Redox Potentials in K53R. Several lines of evidence presented thus far indicate that the redox potential of the FAD in K53R has been raised by the substitution of arginine for lysine. An attempt has been made to measure these potentials directly by equilibrating the enzyme with phenosafranine, a dye of known redox potential. Reduction was accomplished by equilibration with a catalytic concentration of benzyl viologen radical formed by the anaerobic reaction of xanthine oxidase with xanthine. The input of electron equivalents into the enzyme was estimated by the increase in absorbance at 290 nm. This is an isosbestic point in the reduction of phenosafranine under the same experimental conditions, and hence the absorbance increase at this wavelength due to the conversion of xanthine to urate can be used to estimate the number of reducing equivalents delivered to K53R by the xanthine/xanthine oxidase reducing system. In such an experiment (Figure 10), the reduction of flavin was measured at 454 nm after correction for the absorbance decrease due to reduction of phenosafranine, and quantitated by employing the measured extinction change for full reduction of K53R of 8900 M^{-1} cm^{-1}. The difference in total electron equivalents delivered to the enzyme (determined at 290 nm) and the electron equivalents accepted by the flavin (determined at 454 nm) was ascribed to the disulfide. Although the calculated reduction curves of Figure 10 do not show perfect Nernstian behavior, it is clear that both enzyme flavin and disulfide are reduced approximately equipotentially with the phenosafranine, the flavin being reduced slightly adhead of the disulfide. The midpoint potential of the enzyme flavin is estimated as –254 mV and that for the redox-active disulfide as –264 mV. These values should be compared with those of –314 mV for the flavin and –264 mV for the disulfide that have been estimated for the wild-type enzyme (see Discussion).

DISCUSSION

Lipoamide dehydrogenase cycles in catalysis between the E and EH_2 states (Massey et al., 1960). This is shown in Scheme 1, where species I is the oxidized enzyme and species V, IV, III, and II are forms of two-electron-reduced enzyme. In this scheme, the physiological activity (eq 1 in the forward direction) is counterclockwise. The upper sulfur belongs to Cys^{49} and interacts with the flavin while the lower sulfur is from Cys^{44} and is thought to interchange with dihydrolipoamide (Thorpe & Williams, 1976a). Species IV is a thiolate–FAD charge-transfer complex, and species V is its prototropic tautomer (Wilkinson & Williams, 1979). Species III has been observed only with enzyme alkylated on the interchange thiol (Thorpe & Williams, 1976b). The thiolate–FAD charge-transfer complex gives rise to a new band seen as a shoulder on the main flavin band, and is easily quantitated at 530 nm where neither FAD nor $FADH_2$ has absorbance. Species II and species V are not present in detectable amounts in the pig heart enzyme. Thus, species IV is the thermodynamically stable two-electron-reduced species in the enzyme from pig heart (Massey et al., 1960). However, in the enzyme from wild-type *E. coli*, species II, IV, and V constitute 13, 60, and 27% of the EH_2 at pH 7.6 (Wilkinson & Williams, 1979). Whereas in the enzyme from pig heart, neither dihydrolipoamide nor NADH reduces beyond the EH_2 stage at neutral pH, both substrates partially reduce the enzyme from *E. coli* to EH_4. Thus, while it is relatively simple to determine the well-separated E/EH_2 and EH_2/EH_4 redox potentials of lipoamide dehydrogenase from pig heart (–280 and –346 mV, respectively, at pH 7; Matthews & Williams, 1976), these potentials have been estimated with the wild-type enzyme from *E. coli* as approximately –264 and –314 mV, respectively, from data in Wilkinson and Williams (1979), using the difference between the redox potentials, E/EH_2 and EH_2/EH_4, calculated from the formation constant of 50 for comproportionation ($2EH_2 \rightleftarrows E + EH_4$) (Clark, 1960). Since EH_2 is largely a mixture of forms in which the FAD is oxidized and the disulfide is present as the dithiol in enzyme from both species, we associate the E/EH_2 potential with that of the disulfide/dithiol and the EH_2/EH_4 potential with that of the $FAD/FADH_2$. This association of macroscopic redox potentials with specific redox groups is only an approximation but is useful in considering structure–function relationships.

We have sought in this study to compare the ease of reduction of wild-type lipoamide dehydrogenase and K53R in order to establish a qualitative estimate of the relative redox potentials of the FAD in the wild-type and mutant enzymes. In all cases, K53R is more readily reduced. This is of particular interest because of the crucial position of Lys^{53} near the isoalloxazine N5 and the bound NADH pyridinium C4′. This lysine residue is conserved in all homologous enzymes of the pyridine nucleotide-disulfide oxidoreductase family, and it has been suggested that in glutathione reductase it influences the redox potential of the flavin (Pai & Schulz, 1983).

Quantitative assessment of the comparative degree of reduction is made difficult by the fact that the intermediates in the reduction of K53R and wild-type enzyme differ in two notable ways. First, in reductive titrations with dihydrolipoamide or NADH, moderate amounts of neutral semiquinone are produced with K53R but not with wild-type enzyme. Second, an $FADH_2$–NAD^+ charge-transfer complex is stabilized in K53R (species II in Scheme 1). It is characterized by a very broad band maximum around 750 nm (Massey & Palmer, 1962). This band is observed in wild-type enzyme only in the presence of excess NAD^+ and in the absence of NADH (Williams, 1965). The semiquinone, absorbing maximally from 565 to 625 nm (Massey & Palmer, 1966), obscures low levels of thiolate–FAD charge-transfer complex that might be present in K53R. However, it appears that very little if any of this charge-transfer complex is formed. For example, in the photoreduction shown in Figure 9, there is a distinct trough between the semiquinone band and the main flavin band where the thiolate–FAD charge-transfer complex is best observed. This trough is also seen in the titrations with dihydrolipoamide (Figure 5), with NADH at high pH where the $FADH_2$–NAD^+ charge-transfer complex predominates, and with NADH at low pH where both semiquinone and the $FADH_2$–NAD^+ charge-transfer complex are present. Thus, the level of thiolate–FAD charge-transfer complex in K53R must be very low indeed. Wild-type enzyme stabilizes significant amounts of this complex at equilibrium (Wilkinson & Williams, 1979; Williams, 1965).[2]

The degree to which lipoamide dehydrogenase is reduced beyond the EH_2 stage is an indication of the separation of the E/EH_2 and EH_2/EH_4 redox potentials, and for any given reductant, it is an indication of the absolute levels of these potentials. The E/EH_2 redox potential of the enzyme from pig heart is a reasonable approximation of the microscopic redox potential of the disulfide/dithiol, since the thiolate–FAD charge-transfer complex is the only detectable species at the EH_2 level. The EH_2/EH_4 redox potential is an approximation of the $FAD/FADH_2$ microscopic redox potential, since the two potentials are well separated. The E/EH_2 and EH_2/EH_4 redox potentials of the wild-type enzyme from *E. coli* can only be associated approximately with microscopic potentials of flavin or disulfide.

The level of reduction is best assessed by the loss of the main flavin band. It is also evidenced by the loss of the thiolate–FAD charge-transfer complex, at least in the wild-type enzyme. Reduction of K53R with 2.0 equiv of dihydrolipoamide (Figure 5) and wild-type enzyme with 3.6 equiv (Williams, 1965) produces residual extinctions at 455 nm of 4.3 and 5.9 mM^{-1} cm^{-1}, respectively. Complete reduction by dithionite is characterized by a residual extinction of 1.6 mM^{-1} cm^{-1} at 455 nm. Moreover, at this stage of the reduction of wild-type enzyme, the thiolate–FAD charge-transfer complex remains ($\epsilon = 1.2$ mM^{-1} cm^{-1}, pH 7.6) (Williams, 1965). This complex forms rapidly upon reduction with dihydrolipoamide ($\epsilon = 2.7$ mM^{-1} cm^{-1}), decaying slowly to the equilibrium value (Williams, 1976). Dihydrolipoamide has a redox potential of −287 mV (Massey, 1960). APADH, with a redox potential of −260 mV, also reduces K53R to a greater degree than wild-type enzyme. Thus, the absence of thiolate–FAD charge-transfer complex and the greater degree of flavin reduction in K53R indicate that its redox potentials, in particular the flavin potential, are less negative than are those of wild-type enzyme.

An additional line of evidence supports the conclusion that the redox potential has been changed by the lysine to arginine alteration. K53R is inactive under normal assay conditions—high concentration of both substrates. Some activity can be detected at very low concentrations of DL-$Lip(SH)_2NH_2$ in the forward reaction shown in eq 1 and at very low concentrations of β-NADH in the back-reaction. This again shows that the enzyme is more easily reduced to the EH_4 level.

Direct measurement of the K53R redox potentials was effected by equilibration with phenosafranine, a dye of known redox potential, as shown in Figure 10. This indicated that the FAD potential had been raised by about 60 mV, while the disulfide potential was unchanged. This direct measurement together with the several indirect indications that the flavin redox potential had been raised demonstrates the sensitivity of the isoalloxazine ring to its electrostatic environment.

Very tight binding of β-NAD^+ to K53R in the oxidized state is revealed by changes in the flavin visible, fluorescence, and CD spectra (Figures 1–3). This is not observed in the wild-type enzyme. The effect of β-NAD^+ on the back-reaction is maximal at a much lower concentration with K53R than with wild-type. Stimulation of the back-reaction by β-NAD^+ is due to the reoxidation of catalytically inactive EH_4 to active EH_2 (Wilkinson & Williams, 1981), and is effected at a lower concentration in K53R than in wild-type enzyme, indicating tight binding of β-NAD^+ to EH_4 in K53R. At higher concentrations, β-NAD^+ becomes inhibitory due to its binding to oxidized enzyme competing with NADH. Since β-NAD^+ binds more tightly both to oxidized enzyme and to EH_4 in K53R than in wild-type, it is reasonable to assume that this difference also applies at the EH_2 level. It has been suggested that the ammonium group of the lysine residue serves to expel the $NADP^+$ in the reaction catalyzed by the closely related enzyme glutathione reductase (Pai & Schulz, 1983). If this is the case in the back-reaction catalyzed by lipoamide dehydrogenase (eq 1), the guanidinium group must be incapable of performing this role.

The sensitivity of the visible and fluorescence spectra of K53R in the oxidized state to pH (Figures 1 and 3) is not observed with wild-type enzyme. A pK_a of 7.0 is observed for the spectral change. The pK_a of the base (His^{444}) in the oxidized enzyme is much lower than 7.0 (Matthews & Williams, 1976). The imidazole side chain of another histidine residue, His^{321}, is near the flavin, and it is possible that the observed pK_a is a reflection of its ionization.

The profound changes in the properties of lipoamide dehydrogenase effected by the alteration of Lys^{53} to an arginine residue were surprising. It was thought that this minimal modification might effect rather small changes and serve essentially as a control. The significant changes might have been expected, however, since arginine has aromatic character effectively distributing the positive charge over several atoms, while the positive charge in lysine is confined to a single atom (Su & Shafer, 1968). Moreover, arginine to lysine mutations rarely occur in evolution (Dayhoff, 1978). Although arginine has one less methylene than does lysine, the center of the charge should be at approximately the same position provided there is no movement of the polypeptide chain to compensate for the larger arginine residue. The ammonium group of the

[2] In a study of wild-type lipoamide dehydrogenase (Williams, 1965), the enzyme was isolated from a "mutant" *E. coli*, M191-6, which is an acetate auxotroph. The corresponding mutation is not in the lipoamide dehydrogenase gene.

[3] Unpublished results of Shompa Datta, L. David Arscott, George C. Russell, Neigel Allison, John R. Guest, and Charles H. Williams, Jr.

lysine residue forms an ion pair with a glutamate residue (Glu^{194} in the *A. vinelandii* enzyme; Mattevi et al., 1991). The ion pair positions the ammonium function of the lysine residue. Changing this residue (Glu^{188} in the *E. coli* enzyme) to an aspartate (E188D) would be expected to weaken the ion pair interaction. Interestingly, E188D is very easily overreduced.[3]

The properties of the flavin in each flavoprotein are modulated by its specific surroundings (Ghisla & Massey, 1986). In an earlier study, a modification, also in the pyridine nucleotide domain, effected profound changes in the spectral properties of the flavin. An isoleucine was changed to a tyrosine, I184Y. The residue occupying the homologous position in glutathione reductase is a tyrosine. Indeed, in glutathione reductase this tyrosine must move in order for NADPH to bind (Pai & Schulz, 1983). The spectral properties of glutathione reductase differ from those of lipoamide dehydrogenase in three obvious ways. Thus, in glutathione reductase, the visible absorbance is red-shifted, the fluorescence is quenched, and the circular dichroism band at 460 nm is positive rather than negative. I184Y shares each of these properties with glutathione reductase (Meada-Yorita et al., 1991). The subtlety of such modulation is made very clear in these two studies. In the present study, the profound effects on the flavin and on its interaction with bound NAD^+, brought about by the substitution of an arginine residue for Lys^{53}, demonstrate the sensitivity of the flavin to the position and intensity of charges in its immediate environment.

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