Expression of rat liver NAD(P)H:quinone-acceptor oxidoreductase in *Escherichia coli* and mutagenesis *in vitro* at Arg-177

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A prokaryotic expression plasmid, pKK-DT2, containing the cDNA of rat liver NAD(P)H:quinone-acceptor oxidoreductase (EC 1.6.99.2; DT-diaphorase) was constructed and used to transform *Escherichia coli* strain JM109. The rat liver quinone reductase was expressed in strain JM109 and was inducible with isopropyl β -D-thiogalactopyranoside (IPTG). The expressed rat protein was purified by affinity chromatography and had kinetic and physical properties identical with the protein purified from rat liver in that it could utilize either NADH or NADPH as the electron donor and its activity was inhibited by dicoumarol. In addition, we have generated four mutants, Arg-177 $\rightarrow$ His (R177H), Arg-177 $\rightarrow$ Ala (R177A), Arg-177 $\rightarrow$ Cys (R177C) and Arg-177 $\rightarrow$ Leu (R177L), using this expression system. Several of the mutants behaved anomalously on SDS/PAGE, but all of the mutant proteins had the expected M_r as determined by electrospray m.s. These results and those obtained from enzyme kinetic analysis, u.v./visible absorption spectral analysis, and flavin and tryptophan fluorescence analysis of the wild-type enzyme and four mutants indicated that mutations at Arg-177 changed the conformation of the enzyme, resulting in a decrease in enzyme activity. Replacing Arg-177 with leucine altered the protein conformation and decreased FAD incorporation.

INTRODUCTION

NAD(P)H:quinone-acceptor oxidoreductase (EC 1.6.99.2; DT-diaphorase) plays an important role in protecting tissues against the mutagenic, carcinogenic and cytotoxic effects of quinones which occur widely in nature (Ernster, 1987). Protection is accomplished by the unique ability of this quinone reductase to catalyse an obligatory two-electron reduction of several quinones, including vitamin K (e.g. menadione), to hydroquinones with either NADH or NADPH as electron donor (Iyanagi & Yamazaki, 1970; Iyanagi, 1987). This reaction is thought to bypass the semiquinone free-radical state, diminishing the formation of quinone-mediated oxygen free radicals which are generated by redox cycling of semiquinones in the presence of oxygen.

The primary structure of rat liver NAD(P)H:quinone-acceptor reductase has been deduced from cDNA sequences (Robertson *et al.*, 1986; Bayney *et al.*, 1987) and determined by protein microsequencing methods (Haniu *et al.*, 1988). The amino acid sequence of the human enzyme has also been deduced from the cDNA sequence (Jaiswal *et al.*, 1988). This enzyme consists of two identical subunits. Each subunit contains 273 residues and one molecule of the FAD prosthetic group (Hosoda *et al.*, 1974; Rase *et al.*, 1976). Although the primary structure of this quinone reductase has been determined independently by different groups during the last several years, the structure–function relationship remains unidentified.

Previous studies of the rat liver enzyme using affinity-labelling experiments with 5'-fluorosulphonylbenzoyl-adenosine (5'-FSBA) revealed a region of this enzyme, ¹⁴⁶I-T-T-G-G-S-G-S-M-Y¹⁵⁵, that is thought to be a part of the nicotinamide nucleotide-binding site (Liu *et al.*, 1989). Forrest *et al.* (1990) expressed the rat liver enzyme in COS7 cells and initiated site-directed mutagenesis experiments near the proposed nicotinamide nucleotide-binding site. In order to characterize further the structure–function relationship of NAD(P)H:quinone oxidoreductase, we have recently constructed an expression plasmid and succeeded

in expressing the rat liver enzyme in *Escherichia coli*. The expressed enzyme has been purified and characterized. Using this expression system, four mutants, Arg-177 $\rightarrow$ His (R177H), Arg-177 $\rightarrow$ Ala (R177A), Arg-177 $\rightarrow$ Cys (R177C) and Arg-177 $\rightarrow$ Leu (R177L), were produced and their properties examined.

EXPERIMENTAL

Rat liver NAD(P)H:quinone-acceptor oxidoreductase expression in *E. coli*

A *Nco*I/*Hind*III fragment containing the coding region for rat liver NAD(P)H:quinone-acceptor oxidoreductase was isolated from the pCMV-DT plasmid constructed previously (Forrest *et al.*, 1990). This cDNA fragment was ligated into *Nco*I and *Hind*III-restricted prokaryotic expression vector pKK233.2 (Pharmacia, Piscataway, NJ, U.S.A.). The resulting expression plasmid, pKK-DT2, was used to express rat quinone reductase in *E. coli* strain JM109 (Fig. 1).

The conditions for expressing this quinone reductase in *E. coli* are as follows. The expression plasmid pKK-DT2 was transformed into the JM109 strain. 2X YT (2 ml; 1 l of 2X YT contains 16 g of bactotryptone, 10 g of yeast extract and 10 g of NaCl, pH 7.5) containing ampicillin (75 μ g/ml) was inoculated with a single colony of the JM109–pKK-DT2 transformant. After an overnight incubation at 37 °C, the 2 ml culture was used to inoculate 50 ml of prewarmed 2X YT. When the A_{600} of the *E. coli* culture reached 0.6, isopropyl β -D-thiogalactopyranoside (IPTG) was added to a final concentration of 2 mM to induce expression of the quinone reductase. Portions (5 ml) were withdrawn at different time intervals, and quinone reductase activity was determined. For measurement of quinone reductase activity, cells were centrifuged for 15 min at 5000 g, transferred to an Eppendorf tube, washed with 1 ml of phosphate-buffered saline (PBS; contains 8 g of NaCl, 0.24 g of KCl, 1.44 g of Na₂HPO₄ and 0.24 g of KH₂PO₄) (Sambrook *et al.*, 1989) followed by one wash in buffer A (50 mM-Tris buffer, pH 7.5, and 100 mM-NaCl).

Abbreviations used: 5'-FSBA, 5'-fluorosulphonylbenzoyl-adenosine; IPTG, isopropyl β -D-thiogalactopyranoside; PBS, phosphate-buffered saline; TFA, trifluoroacetic acid.

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Fig 1. Structure of pKK-DT2 plasmid

After resuspension in 0.4 ml of buffer A, the cells were disrupted with a sonicator (Branson Sonifier 450, 5×10 s at 20% duty cycle with output-control setting at 4). The sonicated cell mixtures were centrifuged at 13600 g for 10 min. The supernatant was measured for quinone reductase activity.

Purification of the expressed quinone reductase

Transformed JM109 cultures (2 litres) were grown for 17 h in the presence of 2 mM-IPTG, and harvested as described in the previous section. The cells were washed with 200 ml of PBS, followed by 200 ml of buffer A, and resuspended in 60 ml of buffer A. After sonication (3×20 s at 40% duty cycle with output-control setting at 4), the cell debris was removed by centrifugation at 10000 rev./min (13800 g) for 10 min in a Beckman JA-17 rotor. The pellets were resuspended in 60 ml of buffer A and sonicated again. The supernatants were combined and centrifuged at 40000 rev./min (105000 g) for 90 min in a Beckman 50.2Ti rotor. The supernatant from this centrifugation was applied to a 50 ml Affi-gel Blue (Bio-Rad) column (2.5 cm $\times$ 10 cm) which was equilibrated with 500 ml of buffer A. The elution procedure was basically in accordance with the method described by Prochaska (1988). However, 50% ethylene glycol was omitted from the final elution buffer, and the enzyme was not released from the Affi-gel Blue resin by washing with 20 mM-Tris buffer, pH 10, containing 1 mM-NADH. Therefore the column was washed with 3 bed vol. of 50 mM-Tris buffer (pH 7.5)/0.25 M-sucrose/0.5 M-KCl, which was used in the method described by Sharkis & Swenson (1989). The enzyme was eluted from the column with the latter buffer containing 8 mM-NADH. The active fractions were pooled and concentrated by precipitation with 75% satd. $(\text{NH}_4)_2\text{SO}_4$. The enzyme pellet was resuspended in a small volume of 50 mM-potassium phosphate, pH 7.5, and dialysed against the same buffer. The purified enzyme was stored at -70°C .

Enzyme assay

NAD(P)H:quinone-acceptor oxidoreductase activity was determined spectrophotometrically by measuring the reduction of cytochrome *c* at 550 nm ($\epsilon_{550} = 18.5 \times 10^3 \text{ M}^{-1} \text{ cm}^{-1}$) at 25°C . Unless indicated specifically, the routine assay mixture (1 ml) contained 25 mM-Tris buffer, pH 7.5, 200 μM -NAD(P)H, 0.8 μM -menadione and 30 μM -cytochrome *c*. In this assay, menadione is the electron acceptor, and cytochrome *c* is used to reoxidize the menadiol formed. The reaction was initiated by addition of the enzyme. The assays were performed at least in duplicate. When assays were performed on a crude enzyme preparation, the

activity of the expressed quinone reductase was distinguished from those of other quinone reductases by its sensitivity to treatment with 1 μM -dicoumarol, which completely inhibits it (Ernster *et al.*, 1962). Protein concentrations were determined by the method of Bradford (1976).

Site-directed mutagenesis experiments

The Altered Sites Mutagenesis Kit from Promega Co. (Madison, WI, U.S.A.) was used for the mutagenesis experiments. A HindIII cDNA fragment (1286 bp) containing the coding and 3'-flanking regions of the rat liver quinone reductase was excised from the pCMV-DT plasmid (Forrest *et al.*, 1990) and subcloned into the pSelect-1 plasmid (Promega). On transformation into the JM109 strain, single-stranded DNA was generated by infecting with helper phage R408. Site-directed mutagenesis at Arg-177 was accomplished by annealing the single-stranded DNA with an ampicillin repair oligonucleotide (Promega) and with mixed synthetic primers, 5'-GAAGCCACAGAANNNCAGAATGCCACT-3'. The second strand was synthesized with T4 DNA polymerase and ligated with T4 DNA ligase. The products were transformed into *E. coli* BMH 71-18 MutS strain (a mismatch repair minus strain; Promega). The DNA prepared from ampicillin-resistant overnight culture was then transformed into the JM109 strain and plated on Luria broth plates containing 125 μg of ampicillin/ml. The mutants were further identified by the disappearance of a *Hha* restriction site (GCGC) within the mutated region, CTG^{L176}CGC^{R177}TTC^{F178}. The specific mutants were then identified by direct DNA sequencing. All mutant cDNA fragments were sequenced completely to confirm that there were no other mutations except those designed. The *Nco*I/*Hind*III fragment was excised from each mutant and subcloned into the expression vector, pKK233.2, as described for the construction of the pKK-DT2 plasmid.

Five mutants were generated by the method described above. They were R177H, R177A, R177C, R177L and R177P.

SDS/PAGE

Purified quinone reductase and its mutants were analysed by SDS/PAGE by the method of Laemmli (1970).

Electrospray m.s. analysis of the expressed proteins

The enzymes were loaded on to a microcapillary h.p.l.c. system interfaced with a Finnigan-MAT electrospray ionization source and TSQ 700 mass spectrometer. The h.p.l.c. step was necessary to remove the 50 mM-phosphate buffer in which the enzymes were stored. The microcapillary h.p.l.c. system consisted of an Applied Biosystems micro-syringe pump system 140A (Foster City, CA, U.S.A.), an Applied Biosystems u.v. detector (model 759A) and a 160 $\mu\text{m} \times 30$ cm internal diameter C_{18} microcapillary reversed-phase column (designed and constructed at the Beckman Research Institute of the City of Hope). Solvent flow was split at a ratio 1:50 before the sample injector and column. Solvent A consisted of aq. trifluoroacetic acid (TFA) (0.1%), and solvent B consisted of TFA (0.070%), acetonitrile (90%) and water (10%). The sample solutions (10 μl) were injected on to the column using a 20 μl injection loop on a Rheodyne valve (model 7125). A linear gradient of solvent was formed from 5% B to 100% B in a period of 30 min, and absorbance was measured at 200 nm. Solvent flow through the column and into the electrospray ionization source was approx. 2 $\mu\text{l}/\text{min}$. The electrospray needle was operated with a voltage differential of approx. 4 kV and a sheath flow of 2 μl of 2-methoxyethanol per min. The mass spectrometer was set to scan from m/z 50 to m/z 2000 in 5 s. Data were acquired in centroid mode, and spectra-deconvolution manipulations were accom-

lished with Finnigan-MAT software, on the mass-spectrometer data-system computer.

Spectral analyses

The purified enzymes were dialysed extensively against 50 mM-phosphate buffer, pH 7.5, at 4 °C to remove NADH which was used to elute the enzyme from the Affi-gel Blue affinity column. The u.v./visible spectra of the wild-type and mutated enzymes were recorded using a Hitachi U-3110 spectrophotometer. A Shimadzu RF5000 spectrofluorophotometer was used for fluorescence analysis. The FAD fluorescence of the purified enzymes was quenched and could be detected only under denatured conditions. Tryptophan emission spectra of these proteins were generated by setting the excitation wavelength at 280 nm or 295 nm.

RESULTS

Expression of rat liver NAD(P)H:quinone-acceptor oxidoreductase in *E. coli*

As shown in Fig. 2, the expression of the quinone reductase in *E. coli* strain JM109 transformed with the expression plasmid

Fig 2. Induction of NAD(P)H:quinone-acceptor oxidoreductase by IPTG

The cells were collected, processed and assayed as described in the Experimental section. ●, +IPTG; ○, -IPTG.

Table 1. Purification of the expressed quinone reductase

The crude 40000 rev./min supernatant was obtained from a 2-litre overnight culture of pKK-DT2-transfected *E. coli*.

Step	Volume (ml)	Total protein (mg)	Specific activity (μmol/min per mg)	Purification (fold)	Yield (%)
Crude 40000 rev./min supernatant	250	1050	48.7	1	100
Affi-gel Blue-purified	120	34.5	1265	26	85

pKK-DT2 could be induced by IPTG, and the level of induction was proportional to the length of incubation time up to 17 h. Incubation with IPTG for longer than 17 h resulted in a decrease in the quinone reductase activity. The expressed enzyme could utilize both NADH and NADPH as electron donor and the activities were sensitive to treatment with dicoumarol. Fig. 2 also shows that in the absence of IPTG the quinone reductase activity is much lower. The vector pKK233.2-transformed JM109 strain exhibited insignificant activity (approx. 0–0.018 μmol/min per mg) in both the absence and the presence of IPTG after a 17 h incubation. The expressed wild-type quinone reductase was purified by use of Affi-gel Blue affinity chromatography, as shown in Table 1.

Properties of *E. coli*-expressed rat liver NAD(P)H:quinone-acceptor oxidoreductase and its mutants

The major purpose of developing this *E. coli* expression system is to perform site-directed mutagenesis to study the structure–function relationship of this quinone reductase. The X-ray structure of this enzyme is not yet available. Extensive comparison of the amino acid sequence of the rat liver quinone reductase with those available in GenBank did not reveal any protein having an amino acid sequence significantly homologous with this enzyme. Our affinity-labelling experiments using 5'-FSBA identified a region, ¹⁴⁶I-T-G-G-S-G-S-M-Y¹⁵⁵, which was tentatively assigned as part of the nicotinamide nucleotide-binding site (Liu *et al.*, 1989). On the basis of this and secondary-structure analysis of the amino acid sequence (Forrest *et al.*, 1990), it was concluded that this segment of the protein might be near the region where the pyrophosphate group of the NAD(P)H molecule binds.

This enzyme has an unusual nicotinamide nucleotide-binding site in that it utilizes either NADH or NADPH as electron donor with equal maximal velocities (Ernster *et al.*, 1962). This indicates that the nicotinamide nucleotide-binding site of the enzyme has a structure that can accommodate either NADH or NADPH. The structural difference between NADH and NADPH is the presence of a phosphate group at the 2' position of the ribose of the AMP portion of NADPH. Basic amino acid residues, such as arginine, lysine and histidine, are often found to form hydrogen bonds with the 2'-phosphate group of NADP(H) (e.g. Pai *et al.*, 1988; Scrutton *et al.*, 1990; Bystroff *et al.*, 1990). On the basis of results from our affinity-labelling experiments and our secondary-structure analysis, we thought that Arg-177 might be situated at a loop region and may play a role in the interaction with the 2'-phosphate group of NADPH. A mutation at this position might affect the binding of NAD(H) and NADP(H) differently.

In order to test the hypothesis described above, mutations were generated at Arg-177 using a cassette mutagenesis approach: mutants R177H, R177A, R177C, R177L and R177P were generated. Mutant R177P had no enzyme activity and was not studied further. Mutant R177H was purified in a manner identical with that for the wild-type enzyme. Mutants R177A, R177C and R177L were eluted from the affinity column with a 1–5 mM-NADH gradient in 20 mM-Tris buffer, pH 10.

The purity of *E. coli*-expressed NAD(P)H:quinone-acceptor oxidoreductase and the mutant preparations was analysed by SDS/PAGE. All of the mutant enzymes except R177L displayed a single band on SDS/polyacrylamide gels (Fig. 3). In contrast with the wild-type enzyme and mutant R177H, mutants R177A and R177C migrated faster than the enzyme purified from rat liver. For mutant R177L, one protein band migrated faster than the rat liver enzyme, whereas a second band migrated more slowly and appeared to be a dimer of the enzyme. Reversed-phase h.p.l.c. analysis of all the mutants including mutant R177L

Fig 3. SDS/PAGE of purified NAD(P)H:quinone-acceptor oxidoreductase and its mutants

The purified proteins were examined on an SDS/12%-polyacrylamide gel. A portion (2 μ g) of each purified enzyme preparation was boiled for 2 min after addition of an equal volume of 2 $\times$ SDS/polyacrylamide gel sample buffer [100 mM-Tris buffer, pH 6.8, 200 mM-dithiothreitol, 4% (w/v) SDS, 40% (v/v) glycerol and 0.001% (w/v) Bromophenol Blue] and was loaded on to the gel. The gel was stained with Coomassie Blue (Bio-Rad Brilliant Blue, R-250). Lanes 1–8 are Bio-Rad high- M_r markers (200000, 116250, 87400, 66200 and 45000), mutants R177L, R177C, R177A and R177H, the wild-type enzyme, rat liver enzyme, and low- M_r markers (97400, 66200, 45000, 31000, 21500 and 14000) respectively.

using a Vydac C-18 column resulted in only one protein peak eluted at 60% solvent mixture B (95% acetonitrile/0.01% TFA).

Although SDS/PAGE often fails to yield consistent M_r determinations for proteins of the same size, the markedly different results for these mutants raises questions about the integrity of the expressed proteins. Fortunately, it is now possible to determine protein M_r values accurately by using electrospray m.s. (Fenn *et al.*, 1989). The accuracy of the mass measurement is dependent on the quality of the spectrum and is generally in the range 0.1–0.01%. Mass values obtained by electrospray m.s. analysis for the wild-type enzyme and four mutants included in this study was as follows: the wild-type enzyme, 30854 (calculated M_r , 30846); R177H, 30806 (calculated M_r , 30827); R177A, 30763 (calculated M_r , 30761); R177C, 30790 (calculated M_r , 30793); and R177L, 30835 (calculated M_r , 30803). These values agree within experimental error with the values calculated for the expected structures. Thus, despite the anomalous behaviour on SDS/PAGE, all of the mutants have the expected M_r . The results also indicated that the N-terminus of the *E. coli*-expressed quinone reductase is not blocked by an acetyl group as is the enzyme purified from rat liver. This was confirmed by Edman microsequence analysis (results not shown) of the expressed wild-type enzyme.

For the kinetic studies of the expressed quinone reductase and its mutants, the quinone reductase assays were carried out in triplicate at different concentrations of NAD(P)H (i.e. 10, 20, 40, 100 or 160 μ M), with respect to each concentration of menadione (i.e. 0.2, 0.4, 0.8 and 1.6 μ M). The double-reciprocal plots of the enzyme revealed that the lines at different fixed substrate concentrations were close to parallel (results not shown), indicating that these results agree with a previous report that this quinone reductase catalyses a Ping Pong mechanism (Hall *et al.*, 1972). Secondary plots of intercepts ($1/V_{\max}$) versus the reciprocal of

Fig 4. U.v./visible spectral analysis of expressed quinone reductase and its mutants

—, Wild-type enzyme; ····, R177H; ---, R177A; - - - - -, R177C; — — —, R177L. Inset, FAD fluorescence spectra for the wild-type enzyme and its mutants. The protein concentration of the enzyme solutions was 0.11 mg/ml.

the concentration of fixed substrate were made, and the true K_m and $V_{\max}$ values for NAD(P)H and menadione were derived from these secondary plots. The $V_{\max}$ values for the wild-type enzyme were determined to be 7670 μ mol/min per mg when NADH was used as the electron donor and 5000 μ mol/min per mg when NADPH was used as the electron donor. The K_m values for NADH and NADPH for the wild-type enzyme were 36.2 and 9.5 μ M respectively. The K_m values for menadione (with NADH or NADPH as the electron donor) were 1.38 and 0.48 μ M respectively.

The K_m values for NAD(P)H and menadione for four mutants were very similar to those of the wild-type enzyme, except that the K_m value of NADH for R177A was slightly lower (i.e. 26.7 μ M), that of NADPH for R177H was slightly higher (i.e. 21.9 μ M) and that of menadione (when using NADH as the electron donor) for R177A was slightly lower (i.e. 0.83 μ M) than those of the wild-type enzyme. In contrast with small differences in K_m values, R177H had 30–40%, R177A and R177C had 15–25% and R177L had 2–4% of the wild-type activity. The dicoumarol-inhibition profiles for the wild-type and four mutants were essentially the same. More than 50% inhibition of the enzyme and its mutants was obtained with 10 nM-dicoumarol. Enzyme activity was completely inhibited in the presence of 200 nM-dicoumarol.

NAD(P)H:quinone-acceptor oxidoreductase contains FAD as the prosthetic group. The wild-type quinone reductase, along with mutants R177H, R177A and R177C, displayed typical u.v./visible absorption spectra for flavoproteins (Fig. 4). Mutant R177C contained slightly less FAD than the wild-type enzyme, whereas mutants R177H and R177A contained normal amounts of FAD based on this u.v./visible absorption spectral analysis. Mutant R177L had an unusual absorption spectrum in that typical flavin absorption peaks were not seen (Fig. 4). It absorbed light above 600 nm, resulting in negative absorption between 420 nm and 600 nm when the absorbance at 600 nm was set to zero. In addition, there was a red shift in u.v.-absorption peak for this mutant compared with the wild-type enzyme and the other mutants. This result suggests that mutant R177L contains a low concentration of FAD or that the flavin prosthetic group remains decreased because of the presence of trace amounts of

NADH which may not be completely removed by dialysis. In order to clarify this point, solutions containing the wild-type enzyme or mutants were boiled for 3 min, centrifuged to remove the denatured proteins, and the FAD fluorescence spectrum of the supernatant was determined. Flavin fluorescence (emission maximum 520 nm) was detected with an excitation wavelength of 460 nm. The flavin content in mutant R177L was estimated to be approx. 22% of that of the wild-type enzyme using such analysis (inset, Fig. 4). On the basis of this FAD fluorescence analysis, the protein concentration of mutant R177H was slightly higher than the estimated concentration. The flavin fluorescence is quenched completely for non-denatured enzyme.

DISCUSSION

This paper describes the construction of an expression plasmid, pKK-DT2, and the expression of rat liver NAD(P)H:quinone-acceptor oxidoreductase in *E. coli*. The expression of quinone reductase is IPTG-inducible and is different from the plasmid developed by Ma *et al.* (1990). By using this expression system, approx. 50 mg of the enzyme can be purified from an overnight 3-litre culture. The *N*-terminus of the expressed enzyme is not blocked. Haniu *et al.* (1988) have reported that the *N*-terminus of the rat liver quinone reductase is blocked by an acetyl group. Since the expressed wild-type enzyme is fully active, this post-translational modification is not essential for the catalytic functions of the enzyme. The electrospray m.s. analysis reveals as well that no other post-translational modification takes place during biosynthesis of this quinone reductase in *E. coli*, in that the determined M_r of the enzyme is only 8 mass units higher than the calculated M_r . No additional post-translational modifications were detected for the enzyme purified from the rat liver (result not shown).

The unusual behaviour of the quinone reductase mutants on SDS/polyacrylamide gels would suggest that the mutations R → A, R → C and R → L might alter the conformation of the proteins such that SDS binds very differently, resulting in modification of their migration. It is unusual to find that mutation causes a change in monomer-dimer equilibrium as is the case for R177L shown by SDS/PAGE.

Kinetic analyses revealed that, despite significant differences in V_{max} values, there were only small changes in the K_m values for NADH, NADPH and menadione among the wild-type enzyme and four mutants. It is interesting that mutant R177H has a K_m value for NADPH approximately twice that of the wild-type enzyme, implying that the affinity of NADPH for this mutant is slightly decreased. Human liver NAD(P)H:quinone-acceptor oxidoreductase has a histidine residue at position 177 (Jaiswal *et al.*, 1988). Without a direct comparison, we do not know whether the human liver enzyme has a higher K_m value for NADPH than that of the rat liver enzyme. Such results indicate that Arg-177 is probably not involved in an ionic interaction with the 2'-phosphate group of NADPH.

Although the binding affinities of NAD(P)H and menadione for these mutants seems not to be changed, the differences in V_{max} values indicate that other changes affecting the enzyme activity do occur. Apparently, the arginine residue at position 177 is important for activity because all four mutants have lower activities. U.v./visible absorption spectra of the expressed proteins reveal that the content of FAD prosthetic group in mutant R177L is significantly lower than that in the wild-type enzyme. This result may in part explain why mutant R177L has a very low activity. However, this is not the only reason, as this mutant has only 2–4% of the activity whereas its flavin content is 22% of the wild-type enzyme (Fig. 4). In addition, three other mutants have similar flavin content, but they have only 20–30% of the activity

of the wild-type enzyme. Therefore, mutations at position 177 brought additional change(s) which affect the enzyme activity. It was recently found that tryptophan fluorescence of mutants R177H, R177A and R177C was more intense than that of the wild-type enzyme (results not shown). In addition, there was a blue shift of emission maximum for the tryptophan fluorescence of mutant R177L. The changes in tryptophan fluorescence of the mutants suggested significant conformational change in the protein on mutagenesis. The perturbation of the mutant conformation may alter the migration of mutants on SDS/polyacrylamide gel as discussed above. Arg-177 may be important in forming hydrogen bonds with acidic residue(s) to maintain the correct protein conformation. The blue shift of the tryptophan fluorescence for R177L suggests that the introduction of a leucine residue at position 177 places the affected tryptophan residue(s) in a more hydrophobic environment.

To summarize our results, although Arg-177 of rat liver NAD(P)H:quinone-acceptor oxidoreductase seems not to play a role in nicotinamide nucleotide or quinone binding, this residue may be important in maintaining the structure of this enzyme for proper catalysis. A mutation at position 177 of this enzyme causes protein conformational changes which affect its catalysis. For mutant R177L, the protein conformation is perturbed to such an extent that FAD incorporation is also significantly affected.

NAD(P)H:quinone-acceptor oxidoreductase is an unusual nicotinamide nucleotide-dependent quinone reductase because it can utilize either NADH or NADPH as the electron donor. As indicated in the Results section, it is difficult to design mutants of this quinone reductase to distinguish the nicotinamide nucleotide selectivity without information on its X-ray structure, particularly when the amino acid sequence is distinctly different from that of other nicotinamide nucleotide-dependent enzymes. We have designed mutants relying on information generated from affinity- and photoaffinity-labelling experiments. The success in expressing the enzyme in *E. coli* and purifying the mutant proteins in significant quantities will allow the characterization of these proteins in a more detailed manner. These analyses are important to enable evaluation of our initially stated hypothesis.

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