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On the lack of coordination between protein folding and flavin insertion in *Escherichia coli* for flavocytochrome b_2 mutant forms Y254L and D282N

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Abstract

Wild-type flavocytochrome b_2 (L-lactate dehydrogenase) from *Saccharomyces cerevisiae*, as well as a number of its point mutants, can be expressed to a reasonable level as recombinant proteins in *Escherichia coli* (20–25 mg per liter culture) with a full complement of prosthetic groups. At the same expression level, active-site mutants Y254L and D282N, on the other hand, were obtained with an FMN/heme ratio significantly less than unity, which could not be raised by addition of free FMN. Evidence is provided that the flavin deficit is due to incomplete prosthetic group incorporation during biosynthesis. Flavin-free and holo-forms for both mutants could be separated on a Blue-Trisacryl M column. The far-UV CD spectra of the two forms of each mutant protein were very similar to one another and to that of the wild-type enzyme, suggesting the existence of only local conformational differences between the active holo-enzymes and the nonreconstitutable flavin-free forms. Selective proteolysis with chymotrypsin attacked the same bond for the two mutant holo-enzymes as in the wild-type one, in the protease-sensitive loop. In contrast, for the flavin-free forms of both mutants, cleavage occurred at more than a single bond. Identification of the cleaved bonds suggested that the structural differences between the mutant flavin-free and holo-forms are located mostly at the C-terminal end of the barrel, which carries the prosthetic group and the active site. Altogether, these findings suggest that the two mutations induce an alteration of the protein-folding process during biosynthesis in *E. coli*; as a result, the synchrony between folding and flavin insertion is lost. Finally, a preliminary kinetic characterization of the mutant holo-forms showed the K_m value for lactate to be little affected; k_{cat} values fell by a factor of about 70 for the D282N mutant and of more than 500 for the Y254L mutant, compared to the wild-type enzyme.

Keywords: apoenzyme; flavoenzymes; lactate dehydrogenation; protein folding; recombinant protein; selective proteolysis; site-directed mutants

Flavocytochrome b_2 (L-lactate cytochrome *c* oxido-reductase, EC 1.1.2.3) from *Saccharomyces cerevisiae* catalyzes the oxidation of L-lactate to pyruvate. After FMN reduction by the substrate, electrons are transferred intramolecularly to heme b_2 then intermolecularly to cytochrome *c*, the physiological acceptor in the mitochondrial intermembrane space. Each subunit (M_r 57,000) of the tetramer consists of two structurally and functionally distinct domains: an N-terminal cytochrome b_5-like heme-binding domain and a C-terminal FMN-binding $\beta_8\alpha_8$-barrel (Xia et al., 1987; Xia & Mathews, 1990; Tegoni & Cambillau, 1994a).

The mechanism of L-lactate dehydrogenation has been intensively studied. The reaction is believed to proceed through a carbanion intermediate, produced by abstraction of the substrate α-proton by an enzyme base (for a review, see Lederer, 1991a). Specific mechanistic roles have been assigned to active-site residues in terms of the carbanion mechanism (Lederer & Mathews, 1987) (Fig. 1). These suggestions are now being probed by site-directed mutagenesis, after the *S. cerevisiae* flavocytochrome b_2 gene was expressed first in yeast (Reid et al., 1988), then in *Escherichia coli* (Black et al., 1989). The properties of the Y143F and

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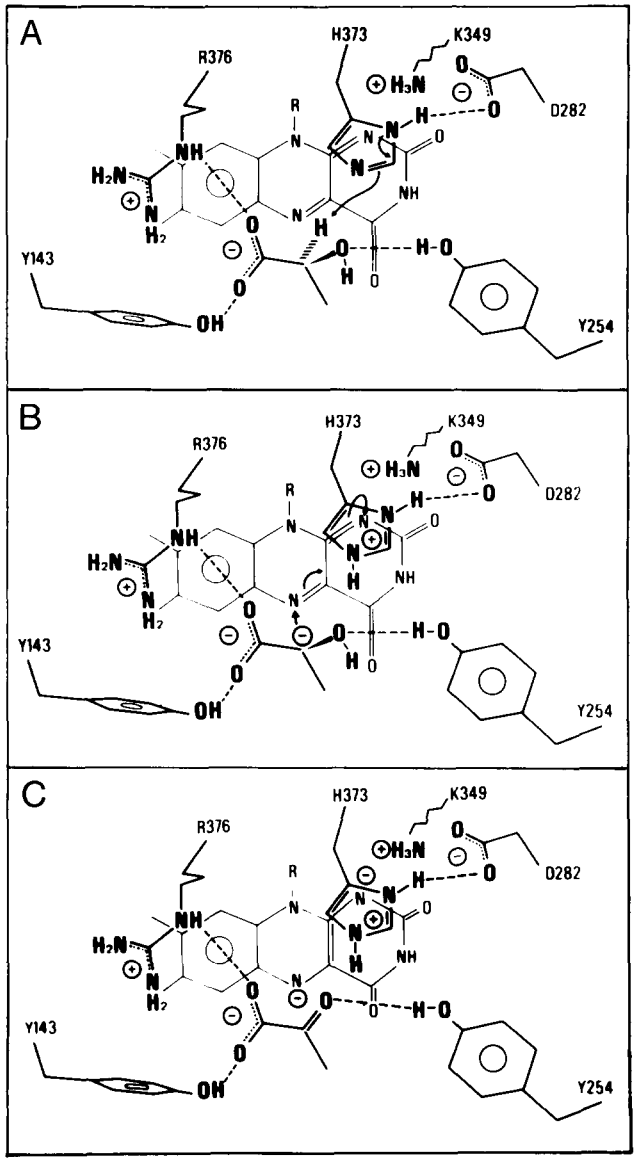

Fig. 1. Proposed mechanism for L-lactate dehydrogenation by flavocytochrome b_2 (updated version of initial proposals [Lederer & Mathews, 1987; Reid et al., 1988]). **A:** Substrate is bound through Y143, R376, and possibly Y254 (see Discussion). The active-site base, H373, removes the substrate α-proton. **B:** An ion pair is formed between the H373 imidazolium and the D282 carboxylate; arrow indicates electron transfer from the carbanion to oxidized flavin but is not meant to indicate how transfer is accomplished. **C:** Putative $E \cdot Fl_{red} \cdot$Product complex. For more complete discussions, see Lederer (1991b, 1992).

Y254F mutant proteins, in particular, were studied in detail (Dubois et al., 1990; Miles et al., 1992; Rouvière-Fourmy et al., 1994). Although their physical properties were not specifically characterized, these mutant enzymes showed no manifest difference with the wild-type protein, either during production in *E. coli* or throughout purification, storage, and handling. We report here that, in contrast, two other mutant enzymes were purified as mixtures of flavohemoprotein and flavin-free hemoprotein. We believe this arises from a defect in flavin insertion during biosynthesis in *E. coli*. These mutant proteins are Y254L

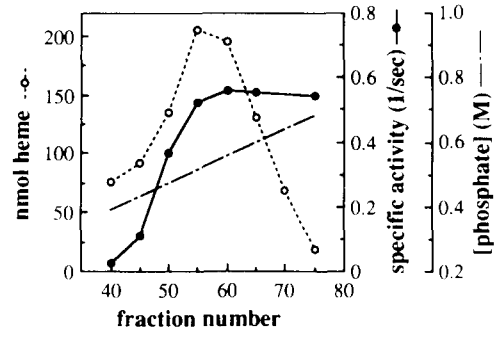

Fig. 2. Chromatography of the Y254L mutant protein on a hydroxyapatite column. After the DEAE-cellulose filtration step (see Materials and methods), 5.4 μmol protein in 0.1 M Na^+/K^+ phosphate buffer, pH 7, was loaded onto a Biogel HTP column (Bio-Rad; 2.5 × 23 cm) eluted at 90 mL/h. The column was developed with the same buffer until absorbance at 280 nm had dropped back to the baseline. The protein was then eluted with a linear gradient between 350 mL of starting buffer and 350 mL of 0.7 M Na^+/K^+ phosphate buffer, pH 7. The usual gradient consists of 1,000 mL starting buffer and 1,000 mL 0.7 M ammonium sulfate in the same buffer (Labeyrie et al., 1978). All buffers contained 20 mM DL-lactate, 1 mM EDTA, 1 mM PMSF. Fractions of 7.1 mL were collected. Specific activity is expressed relative to heme concentration.

and D282N flavocytochromes b_2. Their study was originally initiated in order to probe further the catalytic roles assigned to active-site residues Y254 and D282. The tyrosine was shown to be important for a satisfactory orientation of the α-proton in the transition state, which lies between the Michaelis complex and the carbanion stage; but it was not clear that Y254 did stabilize the Michaelis complex (Dubois et al., 1990). D282 is expected to direct the protonation state of H373 in the oxidized enzyme and to stabilize the incipient imidazolium ion in the transition state (Fig. 1).

Part of the results described here have been presented in preliminary form (Gondry et al., 1994).

Results

Y254L and D282N mutant proteins, as synthesized in E. coli, do not possess a full FMN complement

When partly purified preparations of the Y254L and D282N mutant forms of flavocytochrome b_2 were chromatographed on the standard hydroxyapatite column, the proteins were found to spread on the adsorbent and were eluted only slowly and incompletely. When the ammonium sulfate gradient used to elute the wild-type enzyme was replaced with a steeper gradient of phosphate buffer concentration, the profile shown in Figure 2 was obtained. Protein elution was satisfactory, but the specific activity across the heme elution profile was not constant: the first fractions of the heme-containing peak had low, if any, enzymatic activity. When assayed for flavin, these early fractions were found to have little or no FMN, whereas the late ones with constant specific activity had a flavin/heme ratio of 1. These results showed that the hemoprotein eluted from the column was a mixture of normal enzyme and inactive flavin-free protein. Qualitatively similar results were obtained upon chromatogra-

Table 1. *Purification of Y254L mutant flavocytochrome b_2*

Purification step[a]	Total activity (μmol/min)	Activity yield (%)	Specific activity per heme[b] (s^{-1})	Total hemoprotein (μmol)	OD_{280}/OD_{423}[c]
Total cell extract	174 ± 7	100	–	–	–
40% Ammonium sulfate precipitation (supernatant)	172 ± 3	99	–	–	–
70% Ammonium sulfate precipitation (pellet)	141 ± 1	81	–	–	–
After dialysis	139 ± 1	80	0.42 ± 0.01	5.5	4.4
After DEAE-cellulose	129 ± 3	74	0.41 ± 0.01	5.4	3.6
After hydroxyapatite	120 ± 3	69	0.41 ± 0.02	4.6	0.6

[a] Purification was carried out with 38 g frozen *E. coli* MM294 cells as described in the Materials and methods section and under Figure 2.
[b] Specific activity is expressed as mol of substrate oxidized s^{-1} (mol subunit)$^{-1}$; values represent pooled fractions.
[c] Ratio OD_{280}/OD_{423} is an index of purity; it is 0.5 in the optimal case.

phy of the D282N mutant. Table 1 presents typical purification yields for the Y254L mutant.

The question arose as to whether flavin loss occurred during extraction and purification or whether the prosthetic group had not been totally inserted during protein biosynthesis in *E. coli*. The following relevant observations were made during purification. Addition of 10 μM FMN to enzymatic assays immediately after cell lysis did not increase the activity (in the wild-type enzyme K_D [FMN] = $5 \pm 2 \times 10^{-10}$ M [Iwatsubo & Di Franco, 1965]). No severe activity loss was observed at any specific step during purification. The flavin/heme ratio was 0.6 before chromatography for the preparation described in Table 1, and the specific activity per heme remained constant over the steps where it could be determined, indicating flavin conservation during these steps.

It would thus appear that the flavin/heme stoichiometry of the mutant protein was already low in *E. coli* before extraction. This should not have arisen because of insufficient FMN production by the bacteria because, under identical growth conditions, they produced similar amounts of wild-type flavocytochrome b_2 and of mutant proteins Y143F and Y254F, all of them with a full flavin complement relative to heme. Nevertheless, we tried growing the bacteria in the presence of riboflavin (200 mg/L growth medium) (Bandrin et al., 1979; Shavlovskii et al., 1982), but the mutant holo-enzyme yield was not significantly increased.

Separation of holo-enzyme from flavin-free hemoprotein

Because the activity of the flavin-free protein could not be recovered by simple addition of FMN, we tried to separate inactive from active enzyme. This could be achieved by chromatography on immobilized blue-dextran. Figure 3 shows the separation of two protein peaks upon adsorption of a mixture of D282N flavin-free and holo-proteins onto a Blue-Trisacryl M column after hydroxyapatite chromatography. The first peak (52% yield) had an FMN/heme ratio of 1.0 and a specific activity of 4.21 ± 0.04 mol lactate oxidized per mole enzyme per second. The second peak (30% yield) had an FMN/heme ratio of 0.17 and a specific activity of about 0.7 s^{-1}. A similar fractionation was obtained under the same conditions for the Y254L mutant. Separation between the two forms could also be achieved on the immobilized dye prior to hydroxyapatite chromatography. In that case, some extraneous protein was eluted immediately, but some was also eluted together with the two flavocytochrome b_2 peaks. In order to obtain pure active enzyme, the hydroxyapatite step was then necessary.

It should be noted that the second peak could not be eluted with 10 μM FMN instead of high salt. Pompon and Lederer (1978) showed that deflavocytochrome b_2, which was prepared by rapid gel filtration at acid pH and for which activity could be recovered by FMN addition, was adsorbed on immobilized blue-dextran, whereas the holo-protein was not retarded. The deflavoform could be eluted by addition of salt, ethylene glycol, or a low FMN concentration. It thus appeared that the reconstitutable flavin-binding site was recognized by immobilized blue-dextran. In the present case, because FMN did not displace the flavin-free mutant forms from the immobilized dye, the experiment provided another piece of evidence that the FMN site was nonfunctional in those forms. Their strong adsorption to the column must therefore have been due to other types of interactions.

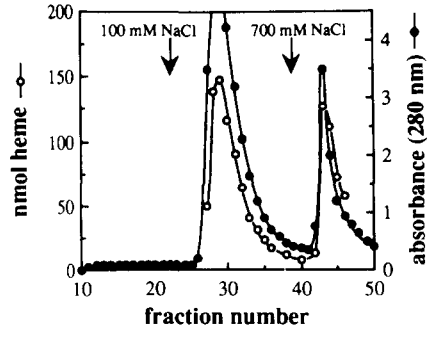

Fig. 3. Separation of D282N holoenzyme from flavin-free protein. A Blue-Trisacryl column (IBF; 2.5 × 15 cm) was equilibrated with 50 mM Na^+/K^+ phosphate buffer, pH 7; 1.25 μmol D282N protein, eluted from hydroxyapatite and dialyzed against 0.1 M Na^+/K^+ phosphate buffer, pH 7, was diluted twice with water before loading onto the column. Elution was then carried out first with 0.1 M phosphate buffer, 10 mM DL-lactate, 1 mM EDTA, 1 mM PMSF, pH 7, then with addition of 100 mM, and subsequently 700 mM NaCl (arrows). Flow rate was 74 mL/h. Fractions of 6 mL were collected.

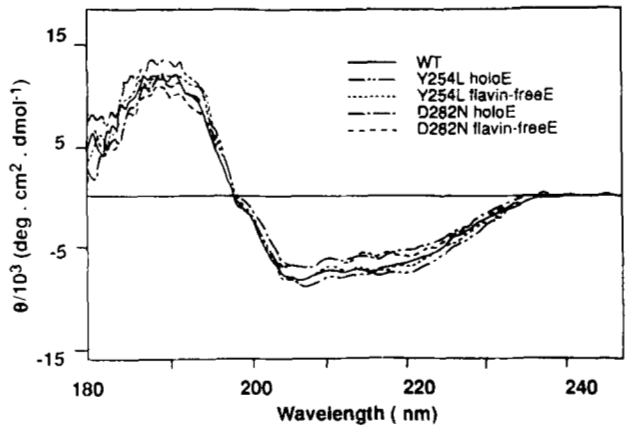

Fig. 4. Far-UV CD spectra of flavin-free and holo-forms of mutants Y254L and D282N. Spectra were recorded in 0.1 M phosphate buffer, 1 mM EDTA, pH 7. Enzyme was in the oxidized state.

The holo- and flavin-free fractions behaved somewhat differently on a Superose 12 column (not shown). The active form showed the expected tetramer as the main constituent (90–95%) with traces of higher molecular weight forms. In contrast, for the flavin-free form, several peaks or shoulders were observed, at elution volumes corresponding approximately to a monomer (15%), a tetramer (62%), an octamer (15%), and higher polymeric species (8%).

Spectral comparison between the mutant flavin-free and holo-enzymes

Figure 4 compares the far-UV CD spectrum of the wild-type holo-enzyme to those of the holo and flavin-free forms of both mutants. Within experimental error, they appear identical and are all comparable to previously published CD spectra of wild-type flavocytochrome b_2 (Iwatsubo & Risler, 1969; Sturtevant & Tsong, 1969; Tsong & Sturtevant, 1969; Jacq & Lederer, 1974). These results suggest that the secondary structure of the mutant proteins is not severely disrupted in the absence of flavin. The flavodehydrogenase domain has most probably assumed the typical $\beta_8\alpha_8$-fold, but local differences must exist to account for the loss of flavin-binding capacity.

Selective proteolysis of mutant flavin-free and holo-enzymes

Flavocytochrome b_2, when purified as originally described by Appleby and Morton (1954), undergoes selective proteolytic cleavage and loses residues 306–310. The derived fragments, α and β, can only be separated under denaturing conditions (Lederer & Simon, 1971). The nicked enzyme is still a tetramer, and this $(\alpha\beta)_4$ form is called the Morton form. Single cleavages in the same area can be obtained with defined proteases under controlled conditions (Fig. 5) (Pompon & Lederer, 1976; Ghrir & Lederer, 1981). Interestingly, this protease-sensitive region corresponds more or less in the three-dimensional structure to a disordered segment (residues 300–316) that is part of a polypeptide excursion between barrel strand β4 and helix α4 (Xia & Mathews, 1990). We have now obtained evidence that in the

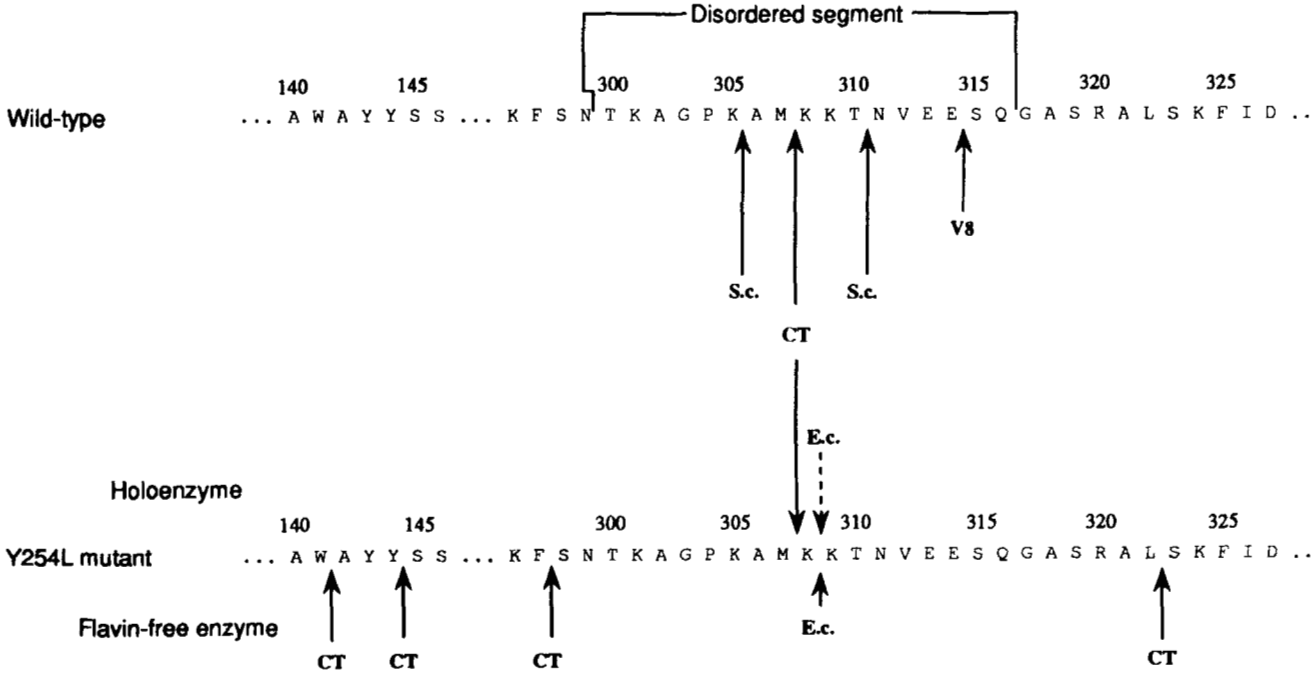

Fig. 5. Cleavage points by proteases along the peptide chain of wild-type and mutant flavocytochrome b_2. Upper line shows cleavage points of the wild-type polypeptide as determined in a previous study (Ghrir & Lederer, 1981). Bottom line shows cleavage points determined in this study for the Y254L mutant (the two forms). "Disordered region" designates the crystallographically invisible segment in subunit S1. In subunit S2, the corresponding segment encompasses positions 300–311 (Xia & Mathews, 1990). E.c., protease from *E. coli*; S.c., protease(s) from *S. cerevisiae*; V8, *Staphylococcus aureus* V8 protease; CT, chymotrypsin. Dashed arrow, a sluggish cleavage (see text).

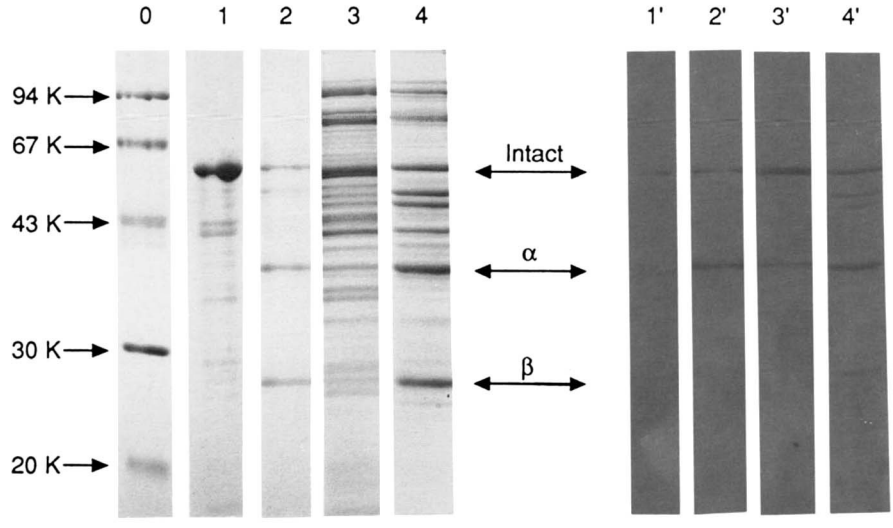

Fig. 6. Correlation between the absence of FMN and the degree of proteolysis in *E. coli* for the Y254L mutant enzyme. Lanes 1–4, Coomassie blue staining; lanes 1′–4′, immunodetection with anti-flavocytochrome b_2 polyclonal immunoglobulins after electrotransfer to nitrocellulose. Lanes 1 and 1′, purified intact wild-type enzyme; lanes 2 and 2′, wild-type enzyme cleaved with chymotrypsin; lanes 3 and 3′, Y254L mutant holo-form separated on blue-dextran-Sepharose; lanes 4 and 4′, flavin-free forms. The two forms of the mutant were produced by the MM294 strain showing proteolytic activity; they were separated from one another on the Blue-Trisacryl column without prior purification on hydroxyapatite, which explains the presence of contaminant proteins on the Coomassie blue-stained gel (see text) (lanes 3 and 4). Immunostaining (lanes 1′–4′) was used to identify the flavocytochrome b_2-derived fragments in the still impure preparation; the experiment was not meant to detect the fragments quantitatively because the concentration range that gives a linear response was not determined; furthermore, unpublished observations indicate that the polyclonal immunoglobulins used in this work react better with fragment α than with fragment β.

Y254L and D282N mutants, the protease-sensitive region reacts differently in the flavin-free and in the holo-enzymes.

The first piece of evidence comes from the observation that upon transformation of a particular colony of *E. coli* MM 294, both mutants were produced in part as "nicked enzymes," yielding the intact subunit as well as α and β fragments upon immunodetection of a crude cell extract. When produced by cells from the same colony, the wild-type enzyme showed only a trace of cleavage. It would thus appear that this specific colony of MM 294 displayed a proteolytic activity that was absent from the other colonies tested. For both purified mutant proteins, the cleavage point was shown to lie between K308 and K309 (Fig. 5). After separation on blue-dextran Sepharose of the holo from the flavin-free form, gel electrophoresis showed that the holo-enzyme had a predominantly intact subunit, whereas the flavin-free protein showed predominantly α and β fragments. Results are presented in Figure 6 for the Y254L mutant, and similar ones were obtained for the D282N enzyme (not shown). These observations suggested that susceptibility to proteolysis in vivo is affected by the absence of the flavin.

This idea was strengthened by proteolysis studies using chymotrypsin at 0 °C, as described before (Pompon & Lederer, 1976). The mutant holo-enzyme yielded the typical pattern of α+β fragments with some residual intact subunit, obtained previously with the wild-type enzyme (Fig. 7). After electrotransfer to an Immobilon membrane, amino acid sequencing of the β fragment showed cleavage to have taken place after M307, as for the wild-type enzyme (Ghrir & Lederer, 1981). In contrast, the flavin-free form showed a more complicated cleavage pattern. The four most abundant fragments (starred in Fig. 7) were analyzed by Edman degradation. The 35-kDa band cor-

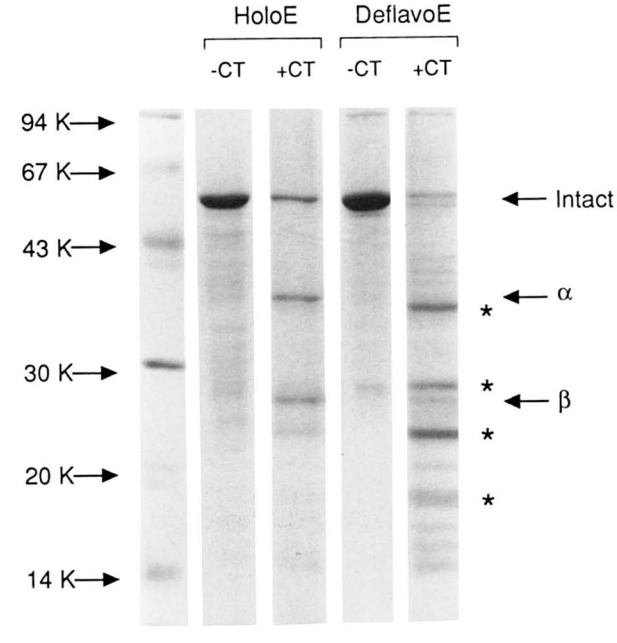

Fig. 7. Comparative chymotryptic digestion of wild-type enzyme and of the holo- and flavin-free forms of the Y254L mutant. As described in the Materials and methods section, 33.5 μM wild-type enzyme, 20.1 μM Y254L holo-enzyme, and 23.3 μM Y254L flavin-free mutant were incubated with chymotrypsin. After 30 min at 0 °C, the reaction was stopped by addition of 0.1 volume of 1 mM PMSF; samples were then submitted to SDS-PAGE. Proteins were stained with Coomassie blue. The left-hand lane corresponds to molecular weight markers. Stars indicate the positions of the protein bands that were submitted to automated Edman degradation, as described in the text.

responded to the N-terminus of the protein. It appeared to be slightly shorter than the normal α fragment (residues 6–307). Its complement was probably the fragment at 28 kDa, which arose from cleavage after F297 (Fig. 5) and was slightly heavier than the normal β fragment (residues 308–511). The next largest fragment, somewhat lighter than the normal β fragment at about 25 kDa, arose from cleavage after L322. It thus probably also extended all the way to the C-terminus. In contrast, the band at about 18 kDa was a mixture of overlapping fragments beginning after W141 and Y144. In view of their size, it is reasonable to assume they extended only up to the central, protease-sensitive region. In summary, the most abundant fragments obtained by proteolysis of the Y254L flavin-free form showed the occurrence of unusual cleavage points at the extreme boundaries of the protease-sensitive region (after F297 and L322) and, even more unexpectedly, at bonds close to Y143, which is an active-site residue (Fig. 1). The gel pattern obtained upon chymotryptic proteolysis of the D282N mutant enzyme was so similar to that observed with the other mutant that no attempt was made to identify the fragments by amino acid sequencing.

Comparative denaturation-renaturation experiments with wild-type and mutant enzymes

The results described in what precedes suggested that part of the mutant polypeptides had acquired, in the cell, an improper conformation for binding flavin. We investigated whether the mutant proteins could be brought back to a structural state that would be competent for binding the prosthetic group. We used a denaturation-renaturation procedure described previously for flavocytochrome b_2 (Mevel-Ninio et al., 1971; Gervais et al., 1980). The first step is a treatment with 6 M guanidine hydrochloride, which leaves the protein colorless. Subsequent removal of the denaturant by dialysis in the presence of the cofactors reportedly leads to an average of 50% activity recovery. Experiments were carried out in parallel with wild-type protein and the two forms of each mutant (see Materials and methods). In all cases, some of the protein precipitated during elimination of the denaturant and the amount of soluble reconstituted hemoprotein ranged from 40 to 50% of the starting material. For the wild-type reconstituted enzyme, the FMN/heme ratio was 0.5 and the specific activity on a per flavin basis was about 80% of the initial value. These results are in keeping with previous ones (Mevel-Ninio et al., 1971; Gervais et al., 1980). In contrast, for none of the mutant forms did the FMN/heme ratio exceed 0.05, and correspondingly no activity could be reproducibly determined. It is probable that a careful selection of experimental conditions would lead to better reconstitution yields (see, for example, Tandon & Horowitz, 1986); nevertheless, these comparative results show that replacing Tyr 254 with Leu and Asp 282 with Asn also influences the folding process of the mutant proteins in vitro.

Preliminary characterization of the holo-forms of the Y254L and D282N mutant enzymes

The visible spectrum of flavocytochrome b_2 is dominated by the heme. Nevertheless, it is possible to observe the FMN spectrum, at least the band centered at 450 nm, by difference spectrophotometry after adding sulfite to the reference of a pair of enzyme-containing cuvettes. The reversible covalent addition of sulfite onto the flavin N5 position induces the appearance

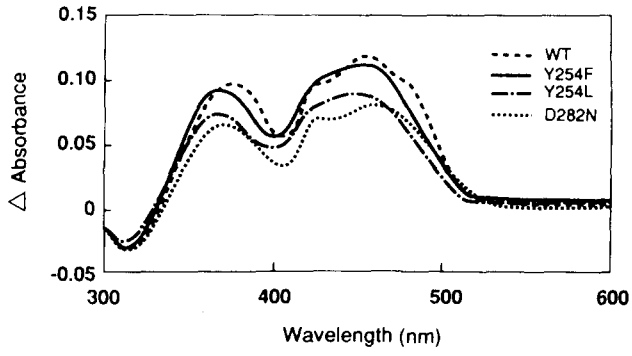

Fig. 8. Sulfite-induced flavin difference spectra. Spectra were recorded at 30 °C. Final sulfite concentration in the reference cuvette was 208 μM, a concentration that is saturating probably only for the wild-type protein (Lederer, 1978; M. Gondry, unpubl. results). Heme concentrations were: 10.4 μM (wild-type), 12.8 μM (Y254F), 10.2 μM (Y254L), and 10.4 μM (D282N). The independently determined FMN/heme ratios were 0.9 for the D282N mutant and 1 for the other proteins.

of a spectrum similar to that of reduced flavin (Massey et al., 1969). Therefore, the difference spectrum is close to that of E·FMNox – E·FMNred because the heme spectrum is unaffected by sulfite. This method has previously been used for monitoring spectral changes induced by ligand binding to wild-type flavocytochrome b_2 (Lederer, 1978). Figure 8 shows the sulfite-induced difference spectra for the wild-type enzyme, for the previously described Y254F mutant enzyme (Dubois et al., 1990), and for the Y254L and D282N enzymes studied in this paper. The wild-type enzyme shows the expected shape with a maximum at 455 nm and prominent shoulders around 420 and 480 nm. The two mutations at position 254 suppress the long-wavelength shoulder, blur the short-wavelength one, and induce a modest hypsochromic shift of the 375-nm band. The D282N mutation also suppresses the long-wavelength shoulder but rather enhances the other shoulder. These observations cannot be interpreted easily. Factors known to influence the electronic spectra of free flavins are, in particular, hydrophobicity and hydrogen bonding. Nevertheless, for protein-bound flavins, the influence of local structural variations or of external agents on the spectral fine structure is not really understood (see, for example, Harbury et al., 1959; Müller et al., 1973). The properties of site-directed mutants may help shed light on this question.

Steady-state rate measurements of ferricyanide reduction by lactate were carried out with the holo-forms of the Y254L and D282N mutant enzymes. The results are compared in Table 2 to the values obtained previously for the wild-type and the Y254F enzymes (Dubois et al., 1990). It can be seen that K_m values are quite similar, whereas k_{cat} values are depressed by the mutations. Leu at position 254 is even less well tolerated than Phe. The significance of these results is discussed below.

Discussion

Y254 and D282 belong to loops 3 and 4 of the barrel, respectively. D282 is normally hydrogen bonded to H373, the active-site base in loop 6, and forms an ion pair with it at some stages of the catalytic cycle. Replacement of D282 by an isosteric Asn

Table 2. *Steady-state kinetic parameters with L-lactate*[a]

Enzyme	K_m (mM)	k_{cat} (s^{-1})[a]	k_{cat}/K_m (10^3 M^{-1} s^{-1})	$\Delta\Delta G^{\ddagger}$[mut-WT] (kcal/mol)
Wild type[b]	0.49 ± 0.10	270 ± 30	551 ± 165	–
Y254F[b]	0.35 ± 0.07	6.10 ± 0.25	17.4 ± 4.2	2.1
Y254L	0.39 ± 0.04	0.51 ± 0.02	1.3 ± 0.2	3.6
D282N	0.73 ± 0.05	3.90 ± 0.10	5.3 ± 0.5	2.8

[a] k_{cat} is expressed as mol of substrate oxidized s^{-1} (mol subunit)$^{-1}$ with ferricyanide as the monoelectronic acceptor (30 °C). $\Delta\Delta G^{\ddagger}$ values are calculated using the k_{cat}/K_m values. Kinetic parameters were obtained by varying lactate concentrations, keeping ferricyanide at 1 mM. This concentration was found to be saturating for the mutant enzymes, as it is for the wild-type form.

[b] Dubois et al. (1990).

would not be expected to introduce any significant structural alteration. The situation for the Tyr to Leu mutation is less clear because Leu is less bulky than Tyr. The two mutant enzymes are less active than the wild-type one (see below for a discussion), but their properties are in qualitative agreement with predictions based purely on mechanistic proposals. Furthermore, the two mutant proteins were not observed to lose flavin and/or activity more easily than the wild-type enzyme during storage and normal handling conditions, with the exception that the D282N enzyme, once purified, tended to lose some FMN upon concentration by ammonium sulfate precipitation. It would thus appear that the mutations have no deleterious effects on the structure of the folded proteins, a proposition that hopefully will be verified by X-ray crystallography.

In contrast, we suggest that the mutations interfere with the folding process in *E. coli* as well as in vitro. Indeed, the flavocytochrome b_2 mutants Y254L and D282N could not be extracted from *E. coli* as fully flavinylated enzymes. Whereas all the soluble immunoreactive protein had incorporated heme, only part of it had also taken up FMN. Depending on the preparation from individually grown *E. coli* MM 294 cultures, the FMN/heme ratio was found to vary from 0.5 to 0.6 for the Y254L mutant and from 0.3 to 0.6 for the D282N mutant. Somewhat smaller ratios were observed when the mutant proteins were expressed in *E. coli* AR 120. As discussed in the Results, it appears unlikely that any flavin loss occurred during purification. Thus, we suggest that it is during biosynthesis in *E. coli* that flavin fails to be incorporated into all the heme-containing mutant peptide chains. Before discussing the possible reasons for this difference with the wild-type enzyme and a number of other well-characterized mutants, it is worthwhile to examine the existing structural evidence concerning the flavin-free forms of the mutants.

What is the structure of the flavin-free mutant enzymes produced in E. coli?

It should first be stressed that these inactive mutant forms cannot incorporate FMN when this compound is added to the solution, contrary to the deflavoenzyme, which can be obtained by flavin removal from wild-type holo-enzyme under mildly acidic conditions (Baudras, 1965; Pompon & Lederer, 1978). Nevertheless, the CD spectra of the two types of flavin-free forms cannot be distinguished from those of the corresponding holo-enzymes and of the wild-type protein (this work and Iwatsubo & Risler, 1969; Sturtevant & Tsong, 1969; Tsong & Sturtevant, 1969). It would appear, therefore, that the overall secondary structure of the $\beta_8\alpha_8$-barrel is maintained in all these forms. Clues as to what changes may occur upon flavin removal are provided by the elucidation of the crystal structures of apoglutathione reductase and apoglycolate oxidase (Schulz & Ermler, 1991; Sandalova & Lindqvist, 1993). The latter case is particularly relevant to the present discussion because flavocytochrome b_2 and glycolate oxidase are homologous (Lindqvist et al., 1991). The apooxidase indeed maintained the $\beta_8\alpha_8$-barrel fold, but the structure was described as "very flexible, non-compact, almost similar to a molten globule." In particular, loops at the flavin-binding end of the barrel had been disordered by prosthetic group dissociation. Also, parts of the extension of the peptide chain outside the barrel, between β4 and α4, a section of which is invisible in the holo-enzyme, were also found to differ somewhat between apo- and holo-enzyme.

In the case of the mutant flavocytochromes b_2, specific differences in conformation or flexibility in the protease-sensitive region are indicated by the differential susceptibility to proteases of the holo and flavin-free mutant forms. Firstly, in vivo, the unidentified *E. coli* protease with tryptic-like specificity attacks the peptide chain between K308 and K309 essentially only in the flavin-free forms. Secondly, with chymotrypsin in vitro, the mutant holoenzymes behave identically to the wild-type protein, being cleaved between M307 and K308. In contrast, this same bond appears much less sensitive to proteolysis in the flavin-free proteins, in which other bonds are cleaved. Two major identified cleavage points lie after F297 and L322, at opposite ends of the peptide chain excursion between the barrel β4 segment and helix α4 (Fig. 9). Other major cleavage points are observed after W141 and Y144 in helix α_C of the crystal structure, before the peptide chain enters the barrel (Fig. 9). This helix is normally rather close to the flavin because Y143 is an active-site residue that forms a hydrogen bond with the substrate carboxylate (Lederer & Mathews, 1987; Miles et al., 1992; Rouvière-Fourmy et al., 1994). Moreover, the two terminal nitrogens in the side chain of R376, another active-site residue, form good hydrogen bonds to the peptide carbonyl group of Y143 and may also interact with the carbonyl oxygen atoms of Y144 and/or S146. Thus, in the flavin-free forms, structural alterations disrupted helix α_C or made it highly mobile, in probable correlation with a different orientation of the arginine side chain at position 376, which belongs to barrel loop 6.

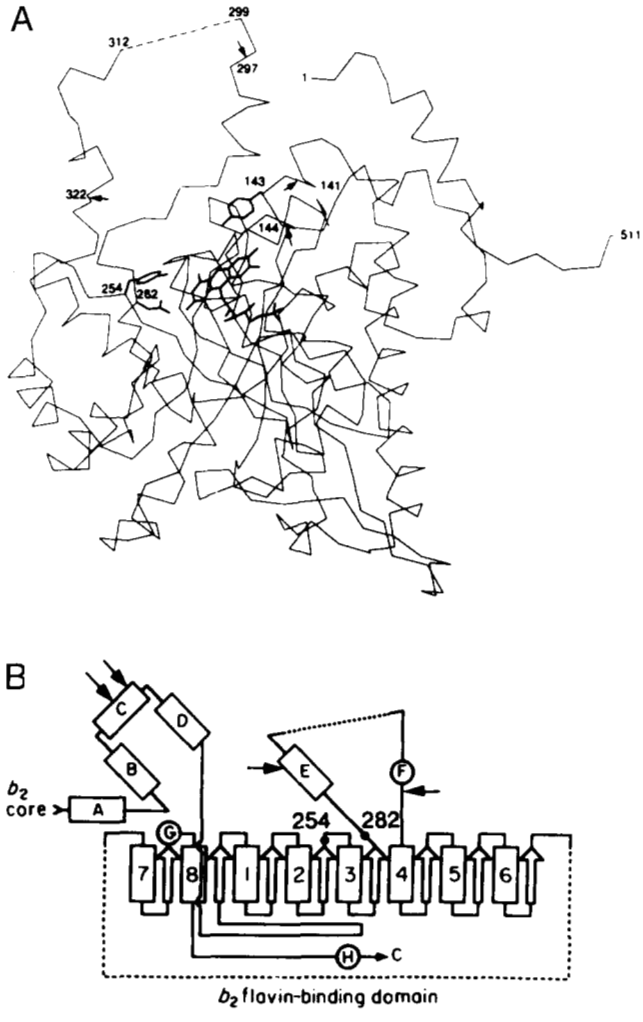

Fig. 9. Chymotryptic proteolysis of the mutant flavin-free forms: location of identified cleaved bonds in the three-dimensional (**A**) and secondary (**B**) structure of the wild-type holo-enzyme. Arrows indicate the cleavage positions identified in the flavin-free Y254L protein. The disordered region (subunit S2) is indicated in dashed lines. The cofactor and side chains of three active-site residues (Y143, Y254, D282) are highlighted by thicker lines. The schematic secondary structure representation in B is taken from Xia and Mathews (1990).

Does loop 4 interact with the active site during catalysis?

Previous proteolysis studies with the wild-type enzyme showed that cleavage at a single bond (chymotrypsin, staphylococcal protease) or at several bonds with peptide loss (yeast proteases) (Fig. 5) always leads to an increase in the substrate K_m value and a decrease in the k_{cat} value (Ghrir & Lederer, 1981). It was noted that this is a surprising situation because one would not expect alterations in a flexible region to entail functional changes, indicative of the existence of some interactions. The situation now appears even more paradoxical because, in the crystal structure, the boundaries of the disordered region lie some 20–25 Å away from the flavin and hence from the active site. More recently, an A306S mutation in loop 4 was also found to have some influence on kinetic parameters (Reid et al., 1988). The present work strengthens the paradox by showing a reciprocal influence between alterations in the FMN-binding site and the conformation or the flexibility of the protease-sensitive loop.

Interestingly, when one superimposes the backbones of glycolate oxidase and flavocytochrome b_2, the observable course of the peptide chain in loop 4 is totally different between positions 296 and 325 (b_2 numbering) (Lindqvist et al., 1991). In the former enzyme, this chain segment covers the C-terminal end of the barrel, whereas in the latter it is further away from the FMN-binding site and abuts the heme-binding domain. The preferentially cleaved bonds after F297 and L322 in the mutant flavocytochrome b_2 flavin-free forms lie at the boundaries of this nonconserved region (Fig. 9). It is thus tempting to suggest that during the catalytic cycle the excursion out of the barrel may change conformation so that residues in the invisible region may somehow interact with the active site, thus competing with the heme-binding domain, which is known to be mobile (Xia & Mathews, 1990). This hypothesis is all the more appealing because many enzymes with the α/β-barrel fold have been demonstrated to possess a flexible loop that interacts with the active site when substrates and/or cofactors bind: triose phosphate isomerase (Joseph et al., 1990), ribulose bisphosphate carboxylase/oxygenase (Lundquist & Schneider, 1989; Knight et al., 1990), and aldose reductase (Borhani et al., 1992), to quote but a few.

On the general problem of recombinant flavoprotein biosynthesis

Biosynthesis of any flavoprotein requires the synthesis of both the peptide chain and the prosthetic group, as well as proper folding of the former and insertion of the latter. Biosynthesis of flavin is constitutive in *E. coli* (Shavlovskii et al., 1982), and this bacterium can meet the demands of highly expressed recombinant flavoproteins (Koyama et al., 1991; Becker et al., 1993; Belmouden & Lederer, 1994). But nothing is known concerning the temporal sequence of these events. It is likely that it may differ depending on the specific fold of individual flavoproteins. For example, when a sarcosine oxidase was expressed to 35% of soluble proteins in *E. coli*, addition of FAD to crude cell extracts increased the specific activity fourfold (Koyama et al., 1991). The case of a bacterial trimethylamine dehydrogenase, which possesses both a covalently bound FMN and a 4Fe-4S cluster, is more similar to that of the flavocytochrome b_2 mutants (Scrutton et al., 1994). The recombinant protein synthesized in *E. coli* showed a full complement of 4Fe-4S cluster, but less than stoichiometric amounts of flavin, and was refractory to complementation by FMN. A possible rationalization of our results is as follows. During biosynthesis in *E. coli*, the peptide chain may start folding up to a point where the scaffolding of the β/α-barrel would be present and the structure would be competent for binding the cofactor. The presence of FMN would induce the final adjustments. In the absence of flavin, the intermediate would collapse to an incompetent state. It can be suggested that the D282N and Y254L mutations would alter the rate of collapse to that state so that this pathway would become competitive with flavin insertion. Our results also suggest that the folding process in vitro is also affected by the mutations.

These considerations quite naturally lead to the question of a possible role of chaperone proteins in the biosynthesis of flavocytochrome b_2 in *E. coli*, similar to what was demonstrated for a number of other proteins (Goloubinoff et al., 1989a, 1989b;

Van Dyck et al., 1989; Lee & Olins, 1992; Hendrick & Hartl, 1993). The evidence concerning flavoproteins is scant. A participation of groES and groEL to flavin insertion in vitro was demonstrated for 6-hydroxy-D-nicotinic acid oxidase (Brandsch et al., 1992). But, in vivo, contrary to the wild-type polypeptide, mutants with Cys → Ser substitutions were found associated with groE proteins (Brandsch et al., 1993). The biosynthesis of plant ferredoxin–$NADP^+$ oxidoreductase in *E. coli* was shown to require the expression of groE genes (Carrillo et al., 1992). For medium-chain acylCoA dehydrogenase, cooverexpression in *E. coli* of homologous groE genes was found to partly rescue into a soluble and active form a mutant with impaired folding and tetramer assembly (Bross et al., 1993). It will be interesting to find out whether, in the presence of additional chaperone molecules, all mutant flavocytochrome b_2 polypeptides can be directed toward the active holo-enzyme, but this issue lies outside the scope of the present paper.

Kinetic characterization of the mutant holoenzymes

The study reported here was undertaken with the aim of clarifying the chemical mechanism of lactate dehydrogenation by flavocytochrome b_2. Detailed properties of the mutant enzymes will be reported later. Meanwhile, a few comments on the first results presented in Table 2 are in order. In the following discussion it will be assumed that K_m values can be equated to K_s values. This has been shown to be the case for the wild-type enzyme and the Y254F mutant form (Dubois et al., 1990). If α-hydrogen abstraction is still the main rate-limiting step in the catalysis by the mutant enzymes Y254L and D282N, a reasonable expectation, then the K_m values for these two proteins can also be considered as K_s values. Neither mutation at position 254 induces a significant change in lactate K_m value. This is unexpected because a hydrogen bond was postulated to exist between the substrate hydroxyl group and the Y254 phenol group (Fig. 1). It was suggested that the aromatic ring of F254 could play an orienting role in the Michaelis complex through weakly electrostatic interactions (Burley & Petsko, 1988; Dubois et al., 1990), and it was to test this hypothesis that mutant Y254L was prepared. The lack of K_m alteration does not support the existence of a hydrogen bond in the ES complex between the substrate and Y254. Yet this is in contradiction with the high K_i value observed for propionate (Genet & Lederer, 1990), a clear-cut case where no hydrogen bond can be formed because of the symmetrical removal of the ligand hydrogen bonding group. A solution to this paradox remains to be found. Compensatory interactions may be formed, for example as the result of a reorientation of the substrate and/or the side chain in the Michaelis complex, even though the crystal structure of the unliganded Y254F protein shows the same position for F254 as for Y254 in the wild-type enzyme (Tegoni & Cambillau, 1994b). In contrast, the low k_{cat} value of the Y254L protein can be more easily rationalized. The postulated hydrogen bond may not exist in the Michaelis complex, but it is present in the transition state because the latter is destabilized by 2.1 kcal/mol in the Y254F mutant enzyme. It is thought that the Tyr 254 phenol group orients, by hydrogen bonding, the position of the substrate C2 substituents so that the α-proton is correctly oriented with respect to the active-site base, His 373. Phenylalanine at position 254 may still have a weak orienting effect due to its electrostatic field. Leucine at this same position cannot have the same influence and indeed k_{cat} is lowered more than 500-fold and the transition state is destabilized by 3.6 kcal/mol.

For the D282N mutant enzyme, no K_m alteration was expected (Fig. 1) and indeed the experimental value may not be significantly different from that of the wild-type enzyme. The k_{cat} value, on the other hand, is more than 100-fold lower than that of the wild-type. But the non-negligible value of the remaining activity (4 s^{-1}) suggests that a hydrogen bond still exists between N282 and H373 N^{δ}, leaving N^{ϵ} unprotonated in the oxidized enzyme and capable of acting as a base. Nevertheless, loss of the wild-type electrostatic interaction between D282 and the incipient imidazolium ion destabilizes the transition state in the mutant enzyme by 2.8 kcal/mol. This conclusion, as well as that concerning mutant Y254L, is valid, again, if in both cases α-proton abstraction is still the rate-limiting step. This question will be addressed in the future.

Materials and methods

DNA manipulation, strains, and growth

Site-directed mutagenesis was performed by the Kunkel method of nonphenotypical selection (Kunkel, 1985) using the oligonucleotides 906C (GTCACTGTGAATGCTCCAAG) and 907C (TACCAACTATTGGTTAACTCT) for the D282N and Y254L mutations, respectively, and the single-stranded plasmid, pGR401 (Reid et al., 1988), as template. The oligonucleotides were prepared by Oswel DNA Service (University of Edinburgh, Edinburgh, Scotland, UK). Mutants were subjected to DNA sequence analysis of the entire flavocytochrome b_2 coding region to ensure the absence of unwanted secondary mutations. The D282N and Y254L mutant sequences were transferred into the expression vector pDSb2 (Black et al., 1989) by replacing the wild-type flavocytochrome b_2 coding sequence. The vector was generally expressed in *E. coli* MM294 (Bachmann, 1987), and sometimes in *E. coli* AR120 (Mott et al., 1985). Standard methods for growth of *E. coli*, plasmid purification, DNA manipulation, and transformation were performed as described in Sambrook et al. (1989).

Enzyme purification and assay

The wild-type protein was purified from *E. coli* MM294 cells grown in LB medium as described in Dubois et al. (1990), using ammonium sulfate precipitation after cell lysis, followed by filtration on a short DEAE-cellulose column then by chromatography on a hydroxyapatite column. For the mutants, the chromatography conditions had to be altered. Details are given specifically in the text or in figure legends. Enzyme stock solutions were kept in the oxidized state at −80 °C until use, in the standard buffer (0.1 M Na^+/K^+ phosphate, 1 mM EDTA, pH 7). Concentrations were expressed relative to one heme using $\epsilon_{413}^{ox} = 129.5\ mM^{-1}\ cm^{-1}$; $\epsilon_{423}^{red} = 183\ mM^{-1}\ cm^{-1}$. Enzyme assays were carried out at 30 °C with a Uvikon 930 spectrophotometer, in standard buffer containing 1 mM ferricyanide and 20 mM L-lactate ($\Delta\epsilon_{420}^{ox\text{-}red} = 1.04\ mM^{-1}\ cm^{-1}$).

Size-exclusion chromatography

Size-exclusion chromatography was carried out with an FPLC system from Pharmacia, at room temperature, using a Super-

ose 12 column (Pharmacia) equilibrated and eluted with 0.1 M phosphate buffer, 1 mM EDTA, pH 7, at 0.5 mL/min. The column was loaded with 20 nmol hemoprotein. Identical results were obtained in the presence or absence of 10 mM DL-lactate.

Flavin determinations

Flavin determinations were carried out with a Perkin-Elmer LS-5 fluorimeter (λ_{ex} = 450 nm; λ_{em} = 520 nm) using the internal calibration method described by Pajot (1976) for tryptophan determinations. An amount of protein containing about 200 pmol heme in 1 mL 6 M guanidinium chloride was used for each determination. Solutions of commercial FMN were used for internal calibration; they were spectrophotometrically titrated beforehand (ϵ_{450} = 12.5 mM^{-1} cm^{-1}).

Denaturation–reconstitution experiments

Denaturation–reconstitution experiments were carried out following Mevel-Ninio et al. (1971) and Gervais et al. (1980). Enzyme samples (about 100 μM) were dialyzed for 24 h at room temperature against denaturation buffer (6 M guanidinium chloride in 0.1 M Na^+/K^+ phosphate, 20 μM EDTA, 0.1 M 2-mercaptoethanol, 1 mM PMSF, pH 7.2). After addition of 500 μM FMN and 300 μM protoheme IX into the dialysis bags, the external medium was changed to 0.15 M Na^+/K^+ phosphate buffer, 25 mM L-lactate, 20 μM EDTA, 1 mM PMSF, 0.1 M 2-mercaptoethanol, pH 7.2. Dialysis was carried out at 4 °C under anaerobiosis for 2 days, then for 1 more day against the same buffer without mercaptoethanol. After centrifugation of the precipitate, heme, flavin, and enzymatic activity were assayed in the supernatant.

Controlled proteolysis

Controlled proteolysis of flavocytochrome b_2 was carried out according to Pompon and Lederer (1976). Incubations were carried out in 0.1 M phosphate buffer, 1 mM EDTA, 10 mM DL-lactate, pH 8 at an enzyme concentration of about 30 μM using a chymotrypsin/substrate ratio of 1/100 (w/w). After 30 min at 0 °C, the reaction was stopped by adding 0.1 vol of aqueous 1 mM PMSF. The samples were then submitted to SDS-PAGE according to Douglas et al. (1979). For western blots, electrotransfer to a nitrocellulose membrane was carried out in the running buffer without SDS, with the semidry apparatus from Millipore (Miniblot-SDE) at 0.2 mA/cm^2 for 35 min. The membranes were then soaked for 45 min in 2% bovine serum albumin (w/v) in 0.1 M Tris-HCl buffer, 0.5% Tween, pH 7. They were then incubated at 37 °C for 1 h in a 1/1,000 dilution (in 0.1 M Tris-HCl buffer) of anti-flavocytochrome b_2 immunoglobulins. Detection was carried out, after a 1-h incubation with a 1/1,000 dilution of sheep anti-mouse immunoglobulins conjugated to alkaline phosphatase (Sigma), by addition of naphthol AS-MX phosphate (Sigma) and fast red TR salt (Sigma). The anti-flavocytochrome b_2 antibodies had been raised after intraperitoneal injection of pure flavocytochrome b_2 into a mouse, using standard immunization protocols. Immunoglobulins were precipitated from ascites fluid at 40% ammonium sulfate saturation and redissolved in 0.1 M phosphate buffer, pH 7.

For Edman degradations, proteins were transferred to Immobilon membranes (Millipore) using the same transfer apparatus as above, in 45 mM Tris/borate buffer, pH 8, without SDS, for 35 min at 0.2 mA/cm^2. Automated amino acid sequencing was carried out with a pulsed liquid-phase sequencer model 477A from Applied Biosystems, following the directions of the manufacturer.

CD spectra

CD spectra were recorded with a Jobin Yvon auto-dichrograph model Mark V at 20 °C under a nitrogen stream. Enzyme concentrations were about 10 μM in 0.1 M phosphate buffer, 1 mM EDTA, pH 7, in cells with a 0.2-mm pathlength.

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