Probing the Active Site of L-Aspartate Oxidase by Site-Directed Mutagenesis: Role of Basic Residues in Fumarate Reduction†

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ABSTRACT: L-Aspartate oxidase is a very particular oxidase which behaves as a fumarate reductase in anaerobic conditions. Its primary and tertiary structures present remarkable similarity with the soluble fumarate reductase (FRD) from *Shewanella frigidimarina* and the flavin subunit of the membrane-bound fumarate reductase from *Escherichia coli* and *Wolinella succinogenes.* This and other extensive similarities are consistent with the idea that a common catalytic mechanism for the reduction of fumarate operates for all members of this enzyme group and that the key residues involved in the substrate binding and catalysis are conserved. This manuscript reports information about the role of these basic residues in L-aspartate oxidase: R290, R386, H244, and H351. By means of site-directed mutagenesis, R290 and R386 are mutated to Leu and H351 and H244 are mutated both to Ala and Ser. H351, H244, and R386 mutants bind substrate analogues with higher dissociation constants and present lower k_{cat}/K_m values in the reduction of fumarate. Therefore, the results indicate that R386, H244, and H351 are important for the binding of the substrate fumarate and may play an important but not essential role in catalysis. R290, on the contrary, is mainly involved in catalysis and not in substrate binding since its mutation abolishes the catalytic activity without lowering the affinity of the enzyme for the substrate. The redox properties of all the mutants are identical to the wild-type. The findings are consistent with a model of L-aspartate oxidase active site based on the hypothesis proposed for the soluble FRD from *S. fridimarina*.

L-Aspartate oxidase (EC 1.4.3.16) (LASPO)[1] is a flavoenzyme containing 1 mol of noncovalently bound FAD/mol of protein. The enzyme is strictly L stereospecific and catalyzes the oxidation of L-aspartate to the corresponding imino acid under both aerobic and anaerobic conditions (*1*) using oxygen as well as fumarate as electron acceptor for $FADH_2$ oxidation. Therefore, the protein can be described as a soluble fumarate reductase (*1*), and it displays strong sequence similarity with the flavoprotein subunits of fumarate reductase (FRD) and succinate dehydrogenase (SDH) (*2*−*4*). Very recently, the three-dimensional structures of LASPO from *Escherichia coli* (*5*) and fumarate reductase from different sources (*6*−*10*) have been resolved revealing that there is, also at the 3D structure level, a remarkable similarity among LASPO, the soluble fumarate reductase from *Shewanella frigidimarina* and the flavin subunit of the membrane-bound fumarate reductase respiratory complex from *E. coli* and *Wolinella succinogenes*. From the comparison, it is evident that both the FAD binding domain and the core of the catalytic domain are conserved. On the other hand, the orientation of the capping domain in LASPO is different from the other fumarate reductase structures and may reflect the open conformation of this protein that has been crystallized in the apoform without any ligand bound to the active site (*5*). The extensive similarities described above are consistent with the idea that a common catalytic mechanism for the reduction of fumarate operates for all members of this enzyme group and that the key residues involved in the substrate binding and catalysis are conserved. In LASPO, they are postulated to be R290, R386, H351, and H244 (5), which correspond to R301, R404. H369, and H257 in FRD from *W. succinogenes*, R402, R544, H504, and H365 in FRD from *S. frigidimarina*, and R287, R390, H355, and H232 in FRD from *E. coli*, respectively. However, interpretations of the *W. succinogenes*, *E. coli*, and *S. frigidimarina* enzyme structures provide different hypotheses concerning the fumarate reduction mechanism. In all cases, an hydride transfer from the reduced flavin to the C2 of fumarate is proposed followed by a proton transfer from an acid catalyst to the C3 of fumarate resulting in the formation of the product succinate. Chemical modification and site-directed mutagenesis studies of the flavoprotein subunit of *E. coli* FRD have identified H232 (H244 in LASPO) as a residue that has a key role in catalysis (*11*). This residue has been proposed as a possible active site acid catalyst for fumarate reduction. However, these findings were not confirmed by the following crystallographic observations (*6*−*10*), and Doherty et al. (*12*) strongly suggest that it may be involved in the binding of the carboxylic group of fumarate. They also propose a similar

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[1] Abbreviations: LASPO, L-aspartate oxidase; FRD, fumarate reductase; SDH, succinate dehydrogenase. Enzymes: L-aspartate oxidase (EC 1.4.3.16); fumarate reductase (EC 1.3.99.1); succinate dehydrogenase (EC 1.3.99.1); triosephosphate isomerase (EC 5.3.1.1); citrate synthase (EC 4.1.3.7); horseradish peroxidase (EC 1.11.1.7).

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Table 1: Oligonucleotides Used as Primers to Generate the Coding Strands of Mutated *nad*B Genes[a]

amino acid substitution	restriction enzyme	primer sequence
H244S	*Eco* 47III	5′-TAATCAGTTC**TC**CCCTAC**A**GC▾GCTATATC-3′
H244A	*Eco* 47III	5′-TAATCAGTTC**GC**CCCTAC**A**GC▾GCTATATC-3′
R290L	*Taq* I	5′-AACTGGCCCCGC**T**▾CGATATTGTCGC-3′
H351S	*Alu* I	5′-GTGCCTGCTGCA**AG**▾**C**TATACCTGCGGT-3′
H351A	*Alu* I	5′-GTGCCTGCTGCA**G**▾**C**TTATACCTGCGGT-3′
R386L	*Tru* 91	5′-CACGGCGCTAAC**T**▾**TA**ATGGCCTCGAAT-3′

[a] The corresponding complementary primers (not shown) were also used to obtain the anticoding strands. The substituted nucleotides are in bold; the newly created restriction sites are underlined; arrows indicate the cutting sites of the listed restriction enzymes.

role for H504 (H351 in LASPO) in contrast with the suggestion of Bamford et al. (*10*), who identify this residue as the active site acid. As far as R290 is concerned, its role represents the major discrepancy between the mechanism proposed by Lancaster et al. for FRD from *W. succinogenes* (*7*) and the one suggested by Taylor et al. in *S. frigidimarina* (*8*). In the first case, this residue is proposed to be involved in binding of the C4 carboxylate group of the substrate and of the water molecule important for catalysis. In the second hypothesis, on the contrary, Arg 504 (R290 in LASPO) is indicated as the acid catalyst that donates the proton to C3 of fumarate and the mutant at this Arg in *S. frigidimarina* resulted to be completly inactive (*12*). A third proposal suggests that the residue may be important for the binding of the substrate or the moving of the product out of the active site (*13*). Finally, as far as we know, no site-directed mutagenesis studies are reported in FRD from any sources on residue corresponding to R386 in LASPO.

The aim of this manuscript is to obtain biochemical data about the role of the conserved basic residues in LASPO: H244, H351, R386, and R290. This is the first time that such a study is carried out for LASPO. Moreover, since LASPO does not contain any cofactor besides FAD, it can be used a simplified model for the study of more intrinsically complex enzymes such as SDH and FRD. The results are in good agreement with the role proposed by Taylor et al. (*8*) and Leys et al. (*9*) for the active site residues of the soluble fumarate reductase from *S. frigidimarina*. In particular, it is suggested that R386, H351, and H244 bind the substrate fumarate through electrostatic interaction with the carboxylate groups and play a significant but not essential role in catalysis. R290, on the contrary, is involved in catalysis and not in substrate binding opposite to what proposed by Lancaster et al. for *W. succinogenes* (*7*) and in keeping with the hypothesis for *S. frigidimarina* (*8*, *9*, *12*).

MATERIALS AND METHODS

Generation of nadB Mutants and Enzyme Purification. E. coli nadB gene, inserted into the *Sma*I/*Bam*HI sites of the expression vector pSU1 (*14*), was submitted to direct mutagenesis using the QuickChange Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA) according to the manufacturer's instructions. The oligonucleotides used to generate *nad*B mutants are listed in Table 1. The mutated plasmids were screened by PCR followed by restriction enzyme digestion, taking advantage of the suitable newly created restriction sites. Each mutant was verified by automated DNA sequencing and then used to transform an *E. coli* strain *omp*T$^-$ (BL21) as previously described (*2*). LASPO wild-type and all the mutants were purified according to the procedure described by Mortarino et al. (*2*). Due to the low activity of the mutants (except for H244A and H244S), the presence of the enzyme during purification was determined by N-terminal sequence analysis using a pulse liquid protein sequencer model 477A (Applied Biosystem, Foster City, CA).

Photoreduction of the Enzyme. Photoreduction of wild-type and mutant LASPO was achieved by irradiating the protein under anaerobic conditions in the presence of 15 mM EDTA, 1 μM 5-deazaflavin-3-sulfonate as catalyst, and 5 μM methyl viologen at 25 °C in 50 mM Hepes pH 8.0 (*4*).

Determination of the Dissociation Constants for FAD by Ultrafiltration. Apo-enzyme was prepared as described in Tedeschi et al. (*15*). After incubation of 2 mL of apo-form with HPLC-purified FAD (2 mol of flavin/mol of protein) in Hepes 50 mM, pH 8.0, at 0 °C plus or minus 10 mM fumarate, the material was concentrated to about 1 mL by centrifugation with an Amicon Centricon 30 at 4 °C. The exact volume and the spectrum of the filtrate and the concentrated material were measured and the K_d calculated from the amount of apo- and holo-enzyme determined using the known absorption coefficients and the total protein content. The results were confirmed by diluting the concentrated material with fresh buffer and repeating the concentration and measurement steps.

Coenzyme Extinction Coefficient. The extinction coefficients for the mutant coenzymes were determined by measuring the change in absorbance after release of the flavin due to the addition of 0.075% SDS to 1 mL of 30 μM holoprotein.

Oxidase Activity Assay. Initial rates of the enzyme-catalyzed oxidation of L-aspartate by 0.24 mM oxygen at 25 °C were measured in a coupled assay by following the oxidation of *o*-dianisidine at 436 nm in Hepes 50 mM, pH 8.0, unless otherwise specified. The reaction mixture contained 20 μM FAD (100 μM in the case of H244A, H244S, H351S, and H351A), 10 μg/mL *o*-dianisidine, 64 μg/mL horseradish peroxidase, and variable amounts of the enzyme and L-aspartate (*1*).

Fumarate Reductase Activity. Fumarate reductase activity was measured in anaerobic conditions by following at 550 nm the reoxidation of benzyl viologen reduced by sodium dithionite as previously described for FRD and SDH (*16*). This assay was carried out in Hepes 50 mM, pH 8, using a final enzyme concentration of 0.34 μM for wild-type, H244A and H244S, and 2.2, 3.7, and 1.18 μM for H351A, H351S and R386L, respectively. The extinction coefficient used for the reduced benzyl viologen at 550 nm was 7800 M^{-1} cm^{-1}.

Ligand Binding. Dissociation constant for ligands were measured spectrophotometrically by addition of small vol-

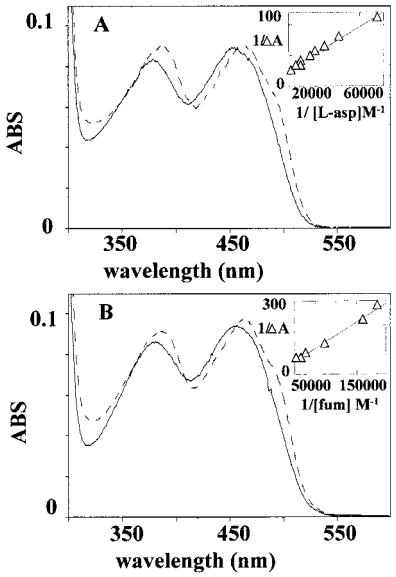

FIGURE 1: Spectra of R290L mutant before and after adding dicarboxylic compounds. (A) R290L spectrum before (—) and after (- - -) addition of 10 mM L-aspartate. (Inset) Benesi-Hildebrand plot (22) for the binding of L-aspartate. (B) R290L spectrum before (—) and after (- - -) addition 2 mM fumarate. (Inset) Benesi-Hildebrand plot for the binding of fumarate.

umes of concentrated stock solutions to samples containing about 10−25 μM holoenzyme at 25 °C. In the case of H351 and H244 mutants, which present a high K_d for the flavin coenzyme, the experiments were carried out after extensive dialysis of the concentrated protein in order to eliminate free FAD.

Determination of the Redox Potential of R386L, R290L, H244A, and H351A. The redox potentials were determined at 25 °C in 8 mM imidazole at pH 7.0 by using anthraquinone-2,6-disulfonate as electron dye (E_{m7} indicator= −0.215 V) as previously described for the wild-type (*4*).

Miscellaneous. Samples for anaerobic experiments were prepared by alternative evacuation and flushing with oxygen-free argon (*17*). All chemicals were of analytical reagent grade. Substrates and substrate analogues were purchased from Sigma-Aldrich. 5-Deazaflavin-3-sulfonate was a generous gift of V. Massey (The University of Michigan, Ann Arbor, MI). Absorption spectra were measured with a Jasco recording spectrophotometer or a Hewlett-Packard 8453 diode array spectrophotometer. Sulfite titration was performed as previously described (*1*).

RESULTS

Enzyme Purification. Typically, around 100 mg of pure enzyme is isolated from 6 L of bacterial growth of wild-type, R386L, and H244A. From the same procedure, approximately 30 mg of R290L, 12 mg of H244S, and 7 mg of H351S and H351A could be prepared.

Characterization of the Mutants. Apart from R386L, the spectral properties of the mutants are very similar to those of the wild-type enzyme (maximum absorbance at 452 nm, $\epsilon = 11\,610\ M^{-1}\ cm^{-1}$) (see Figure 1 as an example). As far as the R386L spectrum is concerned, there is a red shift in the 380 nm region and the peak at 452 nm is partially resolved into a two banded peak with a marked shoulder around 470 nm, suggesting that the enviroment of the flavin is more hydrophobic in this mutant. The extinction coefficients calculated for the FAD−enzyme complexes were 12 400 $M^{-1}\ cm^{-1}$ at 449 nm for R386L, 11 860 $M^{-1}\ cm^{-1}$ at 452 nm for H244A, 11 890 $M^{-1}\ cm^{-1}$ at 449 nm for H351A, and 11 660 $M^{-1}\ cm^{-1}$ at 450 nm for R290L. Opposite to what observed for the wild-type enzyme (*1*), photoreduction of the mutants gives the spectrum of the complete reduced enzyme without blue or red semiquinone formation both in the presence and in the absence of 5 mM succinate (data not shown). Dissociation constants for the FAD−enzyme complex were determined by the ultrafiltration method. As shown in Table 2, in comparison to the wild-type, all the mutants present weaker binding to the flavin cofactor with K_d values ranging from 5.9 to 49 μM. The presence of 10 mM fumarate in the incubation mixture does not significantly stabilize the holoenzyme, opposite to what previously described for the wild-type.

Table 2: Dissociation Constants for FAD−Enzyme Complexes[a]

enzyme	(μM)	+fumarate 10 mM (μM)
wild-type	3.8	0.6
R290L	5.9	11.2
R386L	8.1	5.0
H224A	20.3	12.0
H244S	21.6	15.0
H351S	49.0	32.0
H351A	25.0	28.0

[a] K_d values were calculated by the ultrafiltration method both in the presence and in the absence of 10 mM fumarate.

Table 3: Apparent Kinetic Constants for the Oxidase Activity of the Mutants[a]

enzyme	K_m (mM)	k_{cat} (min^{-1})	k_{cat}/K_m ($min^{-1}\ M^{-1}$)
wild-type	1.6	29.4	18 375
H244A	6.1	6	984
H244S	50	7.5	150
H351A	9.4	0.148	15.7
H351S	11.1	0.016	1.44
R386L	1000	0.15	0.15

[a] Parameters referring to the oxidase activity were calculated by using L-aspartate as electron donor and 0.24 mM oxygen as electron acceptor in 50 mM Hepes, pH 8.0, at 25 °C (*1*).

We checked the L-aspartate oxidase activity of all the mutants at 0.24 mM oxygen and 25 °C. Indeed, apart for R290L mutant which is completely inactive, it appears that the natural substrate is oxidized by the mutants R386L, H244A, H244S, H351A, and H351S with a decrease in the k_{cat}. The calculated values are reported in Table 3. In R386L, the huge increase in the K_m suggests that this residue is involved in substrate binding and the data relative to the binding of dicarboxylic compounds are in accordance with these conclusions. To a lesser extent, the same conclusion is deduced by the increase in K_m due to the substitution of H244 and H351 expecially when the histidine is substituted by the polar residue serine.

Reaction with Sulfite. R290L, H244A, H244S, and H351A bind sulfite with abolition of the visible absorption spectrum of the FAD−enzyme complex. As already observed for the wild-type (*1*), attainment of equilibrium after each sulfite addition is a multiphase process that is complete in about 30 min resulting in K_d values reported in Table 4. Except for H351A, the dissociation constants of the mutants are very

Table 4: Dissociation Constants for the Sulfite-N(5) Flavin Adduct[a]

	wild-type (mM)	R290L (mM)	R386L (mM)	H244S (mM)	H244A (mM)	H351S (mM)	H351A[b] (mM)
	0.010	0.012	n.b.	0.045	0.0036	n.b.	3.2
+succinate	0.0391 (0.0350)	1.9 (2.000)	n.b.	0.123 (0.170)	0.049 (0.038)	n.b.	6.3
+fumarate	0.0442 (0.0373)	1.050 (1.152)	n.b.	0.131 (0.065)	0.118 (0.116)	n.b.	49
+L-aspartate	S	21 (17)	n.b.	S	S	S	S

[a] Aliquots of sufite stock solution were added to LASPO mutants in 50 mM Hepes, pH 8.0, at 25 °C both in the presence and in the absence of various concentrations of fumarate, succinate and L-aspartate, respectively. K_d values were determined from the bleaching at 450 nm. In the presence of the ligands, dissociation constants were calculated from eq 1 and reported in brackets in the table for comparison with the experimental data. n.b. = no bleaching observed at 450 nm, S = there is significant reduction of the enzyme by L-aspartate within the time required to accomplish the experiment. [b] No theoretical values are reported since K_d values for fumarate and for succinate are determined from eq 1 as described in the Results.

Table 5: LASPO Fumarate Reductase Activity[a]

	K_m (μM)	k_{cat} (min^{-1})	k_{cat}/K_m (min^{-1} M^{-1})
wild-type	48	16	333 333
H244A	280	5	17857
H244S	760	2.3	3016
H351A	75	0.3	4000
H351S	94	0.23	2447
R386L	130	0.55	4231
R290L		no activity	

[a] All measurements were made in 50 mM Hepes, pH 8.0, at 25 °C. Fumarate reductase activity was measured as described in the text.

close to the one of the wild-type confirming that the mutations do not alter significantly the binding of the isoalloxazinic portion of FAD. Sulfite titrations of enzyme−fumarate and enzyme−succinate complexes result in a weaker binding of sulfite. The apparent K_d for sulfite adducts determined in the presence of fumarate and succinate are reported in Table 4. The values are in very good agreement with the ones calculated from eq 1:

$$K_{1app} = K_1 + [L_2](K_1/K_2) \quad (1)$$

where K_1 and K_2 are the true dissociation constants for sulfite and fumarate or succinate, respectively, and K_{1app} is the apparent sulfite dissociation constant determined in the presence of a concentration of ligand equal to $[L_2]$. The results suggest that both sulfite and fumarate or succinate may not bind simultaneously to the enzyme irrespective of whether the binding site for the two ligands is the same or different as already reported for the wild-type (*1*). Therefore, it was possible to use the same equation for H351A in order to calculate the K_d values for succinate, fumarate, D-aspartate and L-tartrate which could not be directly determined as described below. In this case, they were calculated from eq 1 using K_{1app} and K_1 measured for sulfite in the presence and in the absence of succinate, fumarate, D-aspartate and L-tartrate, respectively (Table 6); the K_d for oxaloacetate is not reported since sulfite reacts with this ligand in these conditions.

H351S and R386L show a small bleaching of the 450 nm absorbance (8.5 and 4.6% of the initial absorbance in R386L and H351S, respectively) even in the presence of a very large amount of sulfite. These distinct spectral properties suggest that in this case the FAD N-(5)-sulfite adduct formation is hampered by the mutation and sulfite may bind to the protein and not to the flavin opposite to what reported for the wild-type and the oxidases class of flavoenzymes. From the decrease at 450 nm it is possible to calculate a K_d constant of 28 μM for the sulfite−R386L complex. On the contrary, the K_d of the H351S mutant cannot be measured mainly because of the high K_d for the flavin coenzyme and the very small spectral perturbations involved.

LASPO Fumarate Reductase Activity. In ref *1*, we described a new L-aspartate:fumarate oxidoreductase activity of LASPO and a coupled assay set up in order to measure this activity in anaerobic conditions using L-aspartate as electron donor and fumarate as electron acceptor. However, the data reported in Table 3 show that the mutations drastically hamper the L-aspartate oxidase activity of LASPO; therefore, a different method was applied to check the fumarate reductase activity of all the mutants irrespective of the reducing substrate. The reoxidation of benzyl viologen reduced by sodium dithionite in anaerobic conditions was followed at 550 nm as previously described for FRD and SDH (*16*). Control experiments confirm that the assay is reproducible, and the rates are linearly dependent from the amount of the enzyme used (data not shown). As reported in Table 5, it is evident that R290L is completely inactive and all the other mutants present a significant decrease in the catalytic activity in comparison to the wild-type. The results should be compared with the wild-type in terms of k_{cat}/K_m ratios. Using this ratio as a measure of the effectiveness of an enzyme−substrate interaction has the advantage of including only the formation of catalytically productive complexes. Therefore, the results obtained strongly suggest that each mutation hampers the reoxidation of LASPO by fumarate. In the case of H244 and H351, the effects are more prounonced in the Ser than in the Ala mutants in keeping with the results reported above for the oxidase activity. The fact that the fumarate reductase activity is still retained is not consistent with R386, H244, or H351 being essential active site catalyst. Rather we suggest that these basic residues may be important for substrate binding and orientation in keeping with the results reported below. When maleic acid is tested as electron acceptor no activity can be measured for the wild-type and the mutants.

Binding of Dicarboxylic Compounds to LASPO Mutants. As already described by Tedeschi et al. (*1*) and by Mortarino et al. (*2*), LASPO binds dicarboxylic compounds with perturbations of the visible absorption spectrum. The corresponding K_d values are calculated from the spectral changes observed and are reported in Table 6. It should be pointed out that these compounds behave as competitive inhibitors of the LASPO oxidative activity (data not shown). Therefore, these binding studies refer to the active center of LASPO.

As shown in Table 6, the binding of various compounds to R386L, H244A, and H244S is weaker than to wild-type and produces spectral perturbations which are very pronounced in the case of R386. In H244A and H244S, the experiments were carried out both in the presence and in the absence of excess FAD ruling out the possibility that the high K_d of the FAD−enzyme complex may alter the results. Moreover, due to the very slow reactivity of R386L with L-aspartate, it was possible to directly determine the

Table 6: K_d Values for the Binding of Dicarboxylic Compounds[a]

ligands	wild-type (μM)	R290 (μM)	R386 (μM)	H351A[b] (μM)	H244S (μM)	H244A (μM)
L-aspartate	S	50	1560	S	S	S
ϵ (M^{-1} cm^{-1})		4133 (502)	4040 (498)			
D-aspartate	3000	117	20 000	40 000	400	308
ϵ (M^{-1} cm^{-1})	1882 (500)	3657 (507)	4146 (500)		50 (500)	34 (500)
oxaloacetate	60	109	300	n.d.	530	157
ϵ (M^{-1} cm^{-1})	1603 (500)	6624 (500)	3404 (495)		500 (505)	103 (505)
succinate	240	21.6	470	31 000	800	672
ϵ (M^{-1} cm^{-1})	573 (507)	3419 (499)	2711 (490)		341 (500)	110 (505)
fumarate	220	21	1600	2500	2500	317
ϵ (M^{-1} cm^{-1})	1320 (502)	2338 (498)	4733 (498)		635 (495)	296 (500)
L-tartrate	530	109	17 440	20 000	n.d.	1667
ϵ (M^{-1} cm^{-1})	678 (500)	440 (503)	2963 (493)			1170 (500)

[a] All measurements were made in Hepes 50 mM, pH 8.0, at 25 °C. Molar extinction coefficients of the increase in absorbance in the 480−505 nm region are calculated at the wavelength of maximal change indicated in bracket. n.d., not determined, S, substrate. [b] Calculated from sulfite titration experiments by eq 1.

K_d = 1.56 mM for the R386L−L-asp complex (Table 6).

R290L binds oxaloacetate with the same affinity of wild-type and all the dicarboxylic compounds (including L-aspartate and fumarate) much more tightly than the wild-type (Figure 1 and Table 6) ruling out the possibility that the lack of activity described for this mutant is due to the inability to bind L-aspartate or fumarate.

As far as H351 is concerned, it was almost impossible to characterize directly the binding of dicarboxylic compounds to H351A and H351S due to the very small spectra perturbations involved. However, in H351A it was possible to calculate the K_d values from the sulfite titration experiments as reported above. The data suggest that the mutation significantly lowers the affinity of LASPO for substrate analogues (Table 6).

Redox Potential of R386L, R290L, H244A, and H351A. Since the mutations causes a decrease in the catalytic activity of LASPO, we checked the redox properties of the coenzyme in the mutants in order to rule out the possibility that the effect of the mutation is due to a perturbation in the redox potential of flavin rather than to a direct effect on substrate binding and/or catalysis. Moreover, in the case of R386, the comparison among the crystal structure of apoLASPO and holoFRD from different sources (*5*−*10*) suggests that R386 is located near the N-(3)-position of FAD. This observation and the results of sulfite titration prompted us to check if the mutations have an effect on the coenzyme redox potential. To measure the potential of the FAD/$FADH_2$ couple in the mutants, reductive titrations were carried out in the presence of anthraquinone-2,6-disulfonate ($E^\circ = -0.215$ V) as previously described for the wild-type (*4*). The amounts of oxidized and reduced dye and each mutant were quantitated at 330 nm (corrected for the contribution of the coenzyme) and 460 nm (corrected for the contribution of the dye), respectively. The plot log(ox/red) for the enzyme versus the log(ox/red) for the dye gives a 1 unit slope in each case. The values measured for the FAD/$FADH_2$ couple were −0.222, −0.221, −0.200, and −0.221 V for R386L, R290L, H351A, and H244A, respectively. They should be compared with a value of −0.216 V determined for the wild-type and indicate that the mutations do not alter significantly the redox properties of the flavin and its binding site.

DISCUSSION

Although FRD and SDH are well-known enzymes, their catalytic mechanism for the reduction of fumarate and the oxidation of succinate is still debated. In the last 2 years, the determination of the 3D structure of FRD from different sources allowed increased insight into the catalytic process of fumarate reduction and four amino acids were identified as active site residues in this enzyme group: two histidines and two arginines. However, their specific role in the reaction mechanism remains to be conclusively determined. During our studies on LASPO, we collected data confirming that this oxidase can be classified also as a new fumarate reductase (*1*, *4*, *5*, *15*). The aim of this manuscript is to further characterize LASPO from this point of view by means of site-directed mutagenesis of the four amino acids postulated to be in the active site of FRD enzyme family. To explain the results repoted above, it is useful to consider separately the reductive half reaction of LASPO, which involves oxidation of L-aspartate to iminoaspartate and reduction of FAD, and the oxidative half reaction which involves $FADH_2$ oxidation by oxygen or fumarate with the production of hydrogen peroxide or succinate, respectively. In both cases, the catalytic mechanism is still unknown and no information or hypotheses are avaible. In the oxidative half reaction, LASPO is indeed a fumarate reductase when it uses fumarate as electron acceptor. In this manuscript, we focus on this particular aspect in order to find out if the same kind of mechanism proposed for FRD may apply to LASPO as well and if the data reported above may discriminate between the mechanisms proposed for FRD. The results obtained are in good agreement with the one proposed for *S. frigidimarina* (*8*, *9*, *12*) and in Figure 2 an active site model of LASPO based on such proposal is reported. According to this hypothesis, H504 (H351 in LASPO) binds the carboxylic group of fumarate and contributes to delocalize the negative charge in an enolate resonance structure that stabilizes the transient carbanion formed in C3 during fumarate reduction playing a direct role in catalysis. In LASPO, the characterization of the mutants H351A and H351S suggest that H351 may be involved in substrate binding and in catalysis. The study of these mutants was hampered by the high K_d of the flavin coenzyme and the lack of significant spectral variations during titrations with dicarboxylic derivatives. Nevertheless, some conclusions can be drawn from the experiments described above. H351A presents higher K_d values for dicarboxylic compounds in comparison to the wild-type suggesting that this residue has a key role to play in substrate binding. Moreover, the mutation of histidine to alanine and serine affects the overall

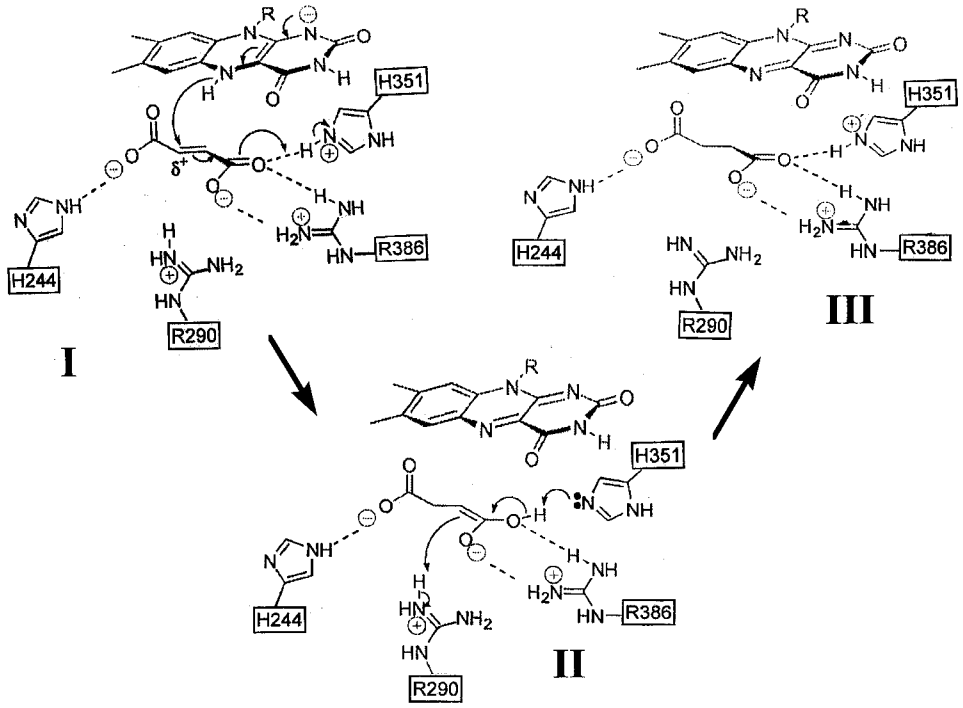

FIGURE 2: Active-site model of LASPO and reaction mechanism for fumarate reduction. The model is derived from Figure 2 in ref *12*.

rate of catalysis when fumarate is used as electron acceptor: 1.9% residual activity in H351A and 1.4% residual activity in H351S if the k_{cat} at pH 8.0 are compared with the values calculated for the wild-type with a small increase of the K_m. These results are comparable with the data reported in ref *12* for the H504 mutant of *S. frigidimarina* where this residue is proposed to be important for Michaelis complex formation. Therefore, we suggest the same role for H351 in LASPO. Besides the fumarate reductase, there are many examples in the literature reporting similar effects upon site-directed mutagenesis of residues which have role analogous to the one reported for H351. For example, in citrate synthase H274 is believed to protonate the enolate to acetylCoA, the mutation in Gly lowers k_{cat} by about 10^3 fold, with little change in K_m (*18*). Similarly, in triose phosphate isomerase H95 is believed to protonate the enediolate intermediate and the substitution of Gln for His leads to little change in K_m while the rate is decreased 140-fold (*19*).

For R 544 (R386 in LASPO), a role in fumarate binding and catalysis is suggested in *S. frigidimarina*. As shown in Figure 2, this Arg binds the substrate and contributes to the polarization of the C2−C3 bond facilitating hydride transfer from N(5) of the reduced flavin to C2. Accordingly, the mutation R386L causes an increase of the K_d values for fumarate and its analogues as well as a significant decrease in the enzyme reactivity toward this electron acceptor (Table 5). In particular, looking at the values of k_{cat} and K_m, it is evident that with a less than 3-fold increase in K_m the mutant is 30-fold less active than wild-type enzyme, suggesting that this residue is involved in substrate binding but is also important for catalysis. Moreover, in the mutant there is only a small decrease of the flavin redox potential in comparison to the wild-type which is very close to the one of free FAD (−0.222 V instead of −0.216 V for R386L and wild-type, respectively) indicating that there is no stabilization of the reduced coenzyme by R386. Therefore, we can suggest that a likely effect of the Arg mutation to Leu may be a less precise positioning of the substrate carboxylate leading to the decrease in k_{cat}. Finally, in wild-type R386 may play a role in the stabilization of the sulfite negative charge since the mutation prevents the formation of the N-(5)-sulfite adduct to the flavin coenzyme.

As far as residue H244 is concerned, in *S. frigidimarina* the corresponding residue H365 is involved in the twisting of the C1-carboxyl group of fumarate out of the plan; therefore, it plays an important role both for the binding and for the activation of the substrate. As shown in Table 6, mutagenesis at this residue results in a weaker binding to dicarboxylic derivatives. Such effect is more evident for H244S mutant than for H244A, supporting the hypothesis that H244 interacts with the substrate carboxylate and that there is repulsion between this group and the hydroxy group of the serine side chain in H244S. However, the poor increase of the K_d values both in H244S and H244A suggests that H244 is not the only residue involved in ligand binding and it may also play a different role in catalysis. In keeping with this there is a significant decrease in the reductive activity of the mutants (14.4% in H244S; 31% in H244A). These effects are similar to those reported above for LASPO mutated at H351 and comparable to the data described in ref *12* for the H365A mutant of *S. frigidimarina* where the residue is proposed to play a role in stabilizing the Michaelis complex. Therefore, we propose the same role for H244 in LASPO.

The major discrepance between the hypothesis proposed by Lancaster et al. (*7*) for *W. succinogenes* and the one suggested for *S. frigidimarina* (*8*, *9*, *12*) concerns the basic residue R290 (in LASPO) which is postulated to be involved in substrate binding in *W. succinogenes* and in proton transfer to fumarate in *S. frigidimarina*. In LASPO, site-directed mutagenesis of this residue provides a clear evidence that R290 plays a pivotal role in catalysis and its mutation abolisches the ability of LASPO to reduce fumarate without hampering the ability to bind such molecule and dicarboxylic

analogues. Moreover, the redox properties of the coenzyme are not altered by the mutation. Remarkably, R290L binds dicarboxylic compounds more tightly than wild-type. In the three-dimensional structure R290 is close to R386 and its substitution with a hydrophobic residue (L) results in the loss of a positive charge. This may result in the decrease of the pK_b of the R386 residue. Therefore, R290L mutant affinity for dicarboxylic compounds increases in keeping with the role proposed for R386. Nevertheless the mutant is completely inactive both in the L-aspartate oxidase and in the fumarate reductase activity, although the enzyme clearly binds L-aspartate and fumarate, implying that R290 plays a role in catalysis as suggested in *S. frigidimarina* (*8*, *9*, *12*) where this residue is proposed to quence the carbanion formed during fumarate reduction. It is higly unusual for an Arg to act as a general acid during catalysis since its pK_a is normally quite high. However, in fumarate reductase, a putative pathway for proton transfer is proposed involving E378 (E260 in LASPO) which forms a bridge between R402 (R290 in LASPO) and R381 (R263 in LASPO) (*20*). This pathway may facilitate the delivery of protons for catalysis in the reduction of fumarate. Since these residues are completely conserved in LASPO as well as in other FRD, we propose that they may play the same role described in ref *20*. Moreover, besides FRD, a role as proton donor for Arg has been already proposed in other enzymatic systems such as the human leukotriene C_4 synthase, where R51 is presumed to open the epoxide ring of leukotriene C_4 by donating a proton (*21*). On the basis of these results and observations we suggest that, besides sharing many biochemical properties (*1*) and similar 3D structure (*5*, *8*, *9*), FRD from *S. frigidimarina* and LASPO may present a common catalytic mechanism for the reduction of fumarate.

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