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FAD Analogues as Mechanistic and 'Binding-Domain' Probes of Spinach Ferredoxin-$NADP^+$ Reductase

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The native flavin, FAD, of spinach ferredoxin–$NADP^+$ reductase, has been replaced by a number of FAD analogues with modifications of the isoalloxazine ring system. The apoenzyme binds 8-mercapto-FAD in its thiolate anion form and 6-hydroxy-FAD in its neutral form. These results are consistent with classification of this enzyme as a dehydrogenase/electron transferase, an ascription originally made on the basis of its physiological function and in common with other properties of this class, e.g. stabilization of the neutral flavin semiquinone.

The chemical reactivity toward methylmethanethiolsulfonate of the 8-mercapto-FAD · enzyme clearly shows that the flavin 8-position is exposed to solvent. On the other hand, the lack of reactivity with the 2-thio-FAD · enzyme indicates that the pyrimidine subnucleus of the flavin is buried within the protein molecule. The seven modified flavins examined all support NADPH–ferricyanide reductase activity, the catalytic velocity being directly proportional to the redox potential of the flavin. No such linear free energy relationship was found between redox potential and activity with ferredoxin or iodonitrotetrazolium as acceptor.

The availability of several new flavin analogues at the FAD level in recent years has been of great advantage in the elucidation of the active site of many flavoproteins [1]. The modified flavins have been used both as probes to examine the influence of the protein on the flavin coenzyme and as a means to investigate solvent accessibility of the protein-bound flavin [1–4]. These studies are particularly useful in the case of flavoproteins where the three-dimensional structure is lacking or is at an initial stage. On the other hand, where the X-ray studies have been carried out at good resolution [5–7], further insights could be obtained by comparison of the structural data with those obtained using flavin analogues. In addition, a comparative examination of the different behaviour towards modified flavins has allowed a more rational subdivision of flavoproteins among the various subclasses [1, 3]. In this regard, 8-chloro, 8-mercapto, 6-hydroxy and 2-thio substituted flavins have been particularly useful [3, 4, 8]. Preliminary to such studies is the availability of a reconstitutable apoprotein of the corresponding flavoenzyme.

Ferredoxin–$NADP^+$ reductase belongs to the class of the flavin dehydrogenases/electron transferases [1]. Many kinetic and physicochemical properties of the enzyme have been investigated [9], whereas sequential and three-dimensional studies are at a very early stage [10]. Up to now, the use of flavin analogues with this enzyme has been hampered by the lack of an easily reconstitutable apoprotein. We recently succeeded in preparing such an apoferredoxin–$NADP^+$ reductase [11]: the result prompted us to investigate the reactivity of flavin analogues with our preparation. In this paper, we report on some properties of apoferredoxin–$NADP^+$ reductase reconstituted with 8-Cl-FAD, 8-mercapto-FAD, 6-OH-FAD, 2-thio-FAD and 1-deaza-FAD. Furthermore, the exposure to solvent of the protein-bound flavin was investigated.

MATERIALS AND METHODS

Ferredoxin–$NADP^+$ reductase from spinach leaves was purified as in [9]. Apoferredoxin–$NADP^+$ reductase was prepared by incubation of holoenzyme with 2.5 M $CaCl_2$ according to the procedure described in [11]. Reconstitution with substituted FAD was accomplished by incubating the apoprotein with an excess of the cofactor at 4 °C.

8-Cl-FAD, 2-thio-FAD, 6-OH-FAD and 1-deaza-FAD were prepared from the corresponding riboflavin derivatives by incubation with the flavokinase/FAD synthetase system of *Brevibacterium ammoniagenes* [12]; 8-mercapto-FAD was prepared by treatment of 8-Cl-FAD with Na_2S as described [13]. 8-Cl-riboflavin was a gift from Dr J. P. Lambooy (University of Maryland); 1-deaza-riboflavin was a gift from Dr E. F. Rogers (Merck, Sharp and Dohme, Rahway, New Jersey) and 2-thio-riboflavin and 6-OH-riboflavin were gifts from the late Professor Peter Hemmerich (University of Konstanz). Methylmethanethiosulfonate was a generous gift from Dr Jules Shafer (University of Michigan). Iodoacetic acid and iodoacetamide were obtained from the Sigma Chemical Co. All other reagents were of analytical grade.

Absorption spectra and kinetics were determined with a Cary 118 or 219 recording spectrophotometer at 4 °C or 25 °C. Activity measurements were performed as in [9, 14]. In reduction experiments, anaerobic 1-ml cells equipped with sidearms were made anaerobic by several cycles of evacuation and refilling with nitrogen purified by storage over Fieser's solution. The NADPH-regenerating system employed in some experiments consisted of the following components in a volume

This paper is dedicated to the memory of Peter Hemmerich whose pioneering work on modified flavins and whose stimulating and provocative discussions over many years have played an important role in the present work.

Abbreviations. Iodonitrotetrazolium, 2-(*p*-iodophenyl)-3-nitrophenyl-5-phenyltetrazolium chloride; Me_2SO_2S, methylmethanethiolsulfonate.

Enzymes. Ferredoxin–$NADP^+$ reductase (EC 1.18.1.2); isocitrate dehydrogenase (EC 1.1.1.42); flavokinase or riboflavin kinase (EC 2.7.1.26); FAD synthetase or ATP:FMN adenylyl transferase (EC 2.7.7.2); superoxide dismutase (EC 1.15.1.1).

of 1 ml: 10 μM NADPH, 2.5 mM DL-isocitrate, 20 μg isocitrate dehydrogenase, 5 mM $MgCl_2$. Isoelectrofocusing was performed on gel slabs using a pH gradient of 4–8 [15]. Activity and protein stainings were carried out as described in [16].

RESULTS

Binding of 8-Mercapto-FAD to Apoferredoxin–$NADP^+$ Reductase and Reduction of the Holoenzyme

On titration of the apoprotein of ferredoxin–$NADP^+$ reductase with 8-mercapto-FAD there is only a minor shift in the absorption spectrum from 520 nm to 548 nm for the protein-bound species, with a shoulder at 470 nm (Fig. 1). The enzyme seems to bind the substituted flavin quite tightly; in fact, the reconstituted protein was chromatographed on Sephadex G-25 to remove the excess flavin before measuring the absorbance. The spectrum remained stable at least for 24 h at 4 °C and a week at −20 °C. The reconstituted 8-mercapto-FAD·enzyme can be easily reduced by NADPH plus regenerating system (see Materials and Methods) (Fig. 1) with the formation of a long-wavelength band absorbing around 720 nm. This form is presumably the neutral radical species, since a similar spectrum was obtained with 8-mercapto-FMN·flavodoxin [2], and since like the neutral radical of FAD in native ferredoxin–$NADP^+$ reductase, it is obtained in a good yield by admitting low quantities of oxygen to the reduced enzyme [17].

From these observations we can conclude that ferredoxin–$NADP^+$ reductase binds the thiolate form of 8-mercapto-FAD; the shoulder at 470 nm is reproducibly present but is not influenced by pH, thus ruling out the possibility of being due to some undissociated 8-mercaptoflavin [2].

Solvent Accessibility of the Flavin Position 8 in Ferredoxin–$NADP^+$ Reductase

By addition of methylmethanethiolsulfonate to the oxidized 8-mercapto-FAD·enzyme, the spectrum is immediately bleached. After addition of dithiothreitol the original spectrum is restored.

The 8-mercapto-FAD·enzyme reacted with iodoacetamide (Fig. 2) at a rate of 77 $M^{-1} \cdot min^{-1}$ at 25 °C, which is faster than with free flavin [3]. Thus, clearly the flavin reacted while bound to protein. In the case of ferredoxin–$NADP^+$ reductase, interference by the modification of sulfhydryl groups of the protein should be negligible. The –SH groups of this flavoprotein are very unreactive towards many sulfhydryl reagent [18]. Specifically the diaphorase activity is not modified by incubation with 10 mM iodoacetamide at 25 °C for 60 min (G. Zanetti, unpublished data).

The 8-SCH_2CONH_2-FAD·enzyme is completely non-fluorescent. Thus, as with the native coenzyme, the fluorescence is fully quenched by the protein. The reconstituted enzyme can be reduced by NADPH plus regenerating system (Fig. 3). Complete reduction can be achieved, leaving a small peak at 420 nm; a long-wavelength band can be seen with a maximum at 620 nm. The latter is typical of the neutral radical species of 8-SR-flavins (V. Massey, unpublished results).

The 8-mercapto-FAD·enzyme also reacted with iodoacetic acid (1.92 $M^{-1} \cdot min^{-1}$ at 10 °C) (data not shown). The spectrum of the product is similar to that of the 8-SCH_2CONH_2-FAD·enzyme with the peak at 491 nm instead of 487 nm.

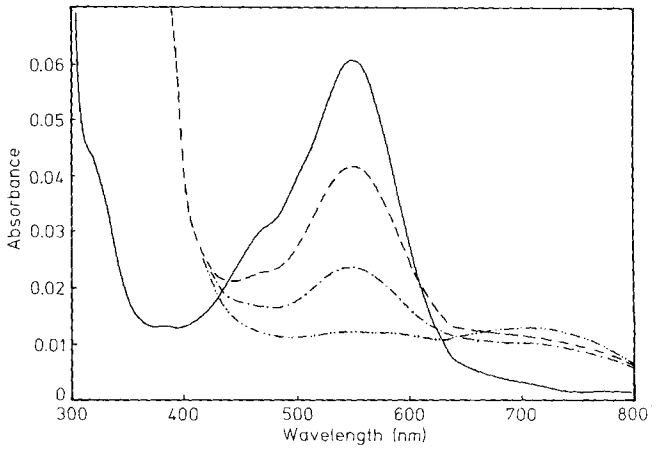

Fig. 1. *Effects of NADPH reduction on ferredoxin-$NADP^+$ reductase whose native flavin has been replaced by 8-mercapto-FAD.* The reconstituted holoenzyme was filtered through Sephadex G-25 equilibrated in 0.05 M Tris/HCl pH 7.4 (25 °C), before taking the spectrum. (——) Oxidized enzyme after the anaerobiosis was established; (– – –) 8 min after addition of a NADPH-regenerating system; (–·–·–) 25 min; (–··–··–) 75 min

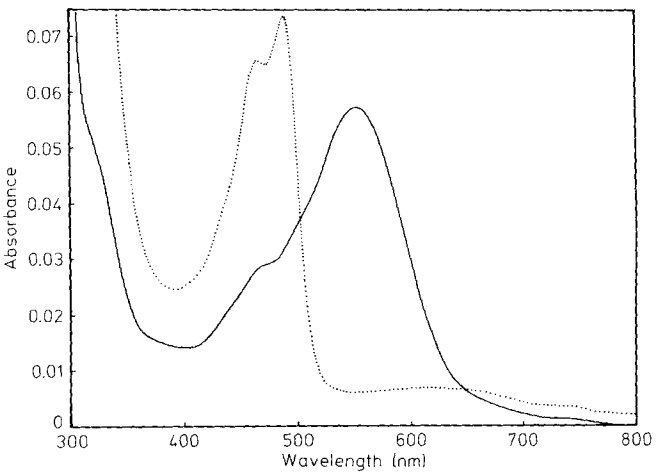

Fig. 2. *Conversion of 8-mercapto-FAD · ferredoxin–$NADP^+$ reductase to 8-SCH_2CONH_2-FAD · ferredoxin–$NADP^+$ reductase by the action of iodoacetamide.* (——) Oxidized 8-mercapto-FAD·enzyme in 0.05 M Tris/HCl, pH 7.4, (25 °C); (·····) about 30 min after 10 mM ICH_2CONH_2 was added

The effect of Na_2S on the 8-Cl-FAD·enzyme was also studied. No reaction was found over a 30-min period. This contrasts strongly with the very rapid reaction of the 8-mercapto-FAD·enzyme with Me_2SO_2S and iodoacetamide. However, there are several examples of this type of behaviour [3], which implies either a barrier to forming the tetrahedral intermediate involved in these nucleophilic displacements reactions, or to charge repulsion by the protein of the nucleophilic anion. The latter hypothesis should be discarded on the basis of the reaction of the 8-mercapto-FAD·enzyme with the iodoacetate anion.

Catalytic Properties of Ferredoxin–$NADP^+$ Reductase Reconstituted with FAD Analogues Modified at Position 8

The introduction of a chlorine atom in the 8-position of FAD seems to boost the diaphorase activity of ferredoxin–

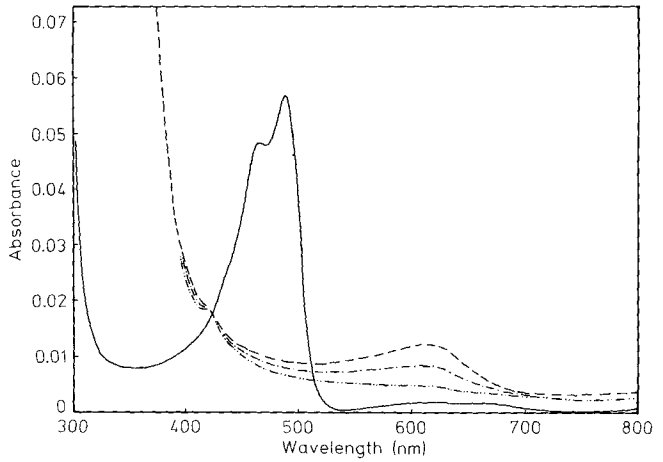

Fig. 3. *NADPH reduction of 8-SCH_2CONH_2-FAD · ferredoxin–$NADP^+$ reductase.* The 8-SCH_2CONH_2-FAD · enzyme, obtained by iodoacetamide treatment of 8-mercapto-FAD · enzyme, was dialysed against 0.05 M Tris/HCl, pH 7.4 (25 °C) at 4 °C. (——) Oxidized enzyme; (– – –) 2 min after addition of a NADPH-regenerating system; (–·–·–) 10 min; (–··–··–) 30 min

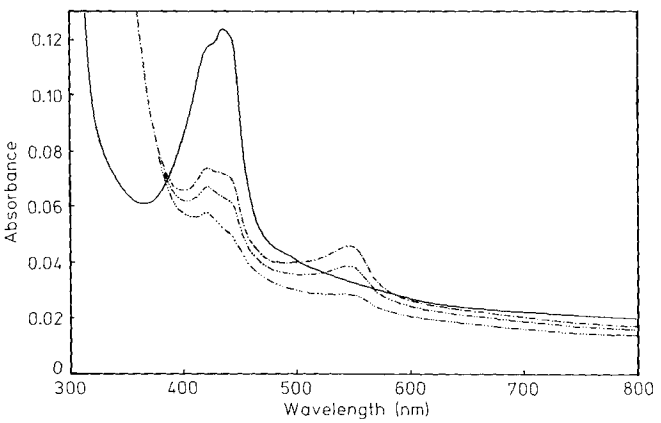

Fig. 4. *NADPH reduction of 6-OH-FAD · ferredoxin–$NADP^+$ reductase.* (——) 6-OH-FAD · enzyme after the anaerobiosis was established; (–·–·–) 2 min after a NADPH-regenerating system was added; (–···–···–) 10 min; (–·····–·····–) 47 min

$NADP^+$ reductase not only with ferricyanide (see Table 1) but also with iodonitrotetrazolium. Instead the substitution with a mercapto group in the same position yields an enzyme with very low activity in all the assays tested. Interestingly, alkylation of the thiolate form by iodoacetic acid produced an enzyme with higher diaphorase activity and, what is more important, which has regained activity with ferredoxin.

Binding of 2-Thio-FAD by Apoferredoxin–$NADP^+$ Reductase: Solvent Accessibility of Position 2, Reduction by the Substrate and Catalytic Properties of the Reconstituted Enzyme

The apoprotein of ferredoxin–$NADP^+$ reductase binds 2-thio-FAD quite readily. After gel filtration to remove the excess flavin, the reconstituted holoenzyme shows a spectrum only slightly changed with respect to free 2-thio-FAD [4]. The binding to the protein produces a red shift of peaks from 317 nm to 325 nm and from 390 nm to 405 nm; the major peak at 488 nm is also red-shifted to 495 nm and a shoulder at 520 nm develops.

The exposure to solvent of the 2-position of FAD can be checked by reacting the reconstituted 2-thio-FAD · enzyme with excess methylmethanethiolsulfonate. The free 2-thioflavin anion reacts readily with this thiol reagent to yield the corresponding 2-$SSCH_3$-flavin disulfide; this reaction is accompanied by a substantial change in the visible absorbance spectrum [4]. Thus, the effect of Me_2SO_2S on the 2-thio-FAD · enzyme, under non-denaturing conditions, was followed by monitoring the spectral changes induced at 520 nm. There was only a very slow decrease of absorbance of the 500-nm absorption band which became evident only after several days.

The reduction by NADPH plus regenerating system is very rapid to give an almost complete bleaching of the 500-nm band. During the reduction, and on admitting air, transient long-wavelength absorption can be observed. This seems to be due to weak charge transfer absorbance between oxidized enzyme and NADPH, and between reduced enzyme and $NADP^+$ respectively, rather than to a radical species; in fact, the latter should have a pronounced absorbance maximum at 680 nm, as judged from experiments with 2-thio-FMN · flavodoxin (V. Massey, unpublished).

The reconstituted 2-thio-FAD · enzyme is catalytically very efficient at least with potassium ferricyanide (Table 1); a ninefold increase is observed, whereas the activity with iodonitrotetrazolium is slightly higher than that of native enzyme. The reaction with ferredoxin is difficult to interpret (see later).

Binding of 6-Hydroxy-FAD to Apoferredoxin–$NADP^+$ Reductase: Reduction by the Substrate and Catalytic Properties of the Reconstituted Enzyme

In order to determine the effects of the hydroxy group at position 6 on the properties of the holoenzyme, we studied the binding of 6-OH-FAD to the apoprotein of ferredoxin–$NADP^+$ reductase. When 6-OH-FAD is added to the apoprotein at pH 7.5, the 423-nm absorption maximum is slightly red-shifted to 435 nm, whereas the 320-nm and 600-nm bands disappeared. The spectrum of the 6-OH-FAD-reconstituted enzyme (Fig. 4) is very similar to the spectrum of free 6-OH-FAD at pH 5.4 [8], suggesting that ferredoxin–$NADP^+$ reductase binds the neutral form of this substituted flavin. Unlike the spectrum of the latter, the spectrum of the 6-OH-FAD · enzyme does not change by varying the pH up to 9.1. Only above this value were the changes consistent with ionization. Accordingly, the pK for the dissociation of the 6-OH-FAD to the anion form has been shifted from 7.1 [8] to a much higher value. The pK of the enzyme-bound form has not been determined accurately, due to possible protein denaturation at the high pH values required. However, the spectrum at pH 10.5 which corresponds roughly to that which would be expected for half ionization, is readily reversed on lowering the pH, even after standing at 0 °C for 12 h.

The 6-OH-FAD-reconstituted enzyme is easily reduced by NADPH plus regenerating system (Fig. 4). The presence of oxygen caused a rapid generation of a 550-nm peak. By analogy with 6-OH-FMN · electron-transferring flavoprotein [8], this intermediate should be the neutral semiquinone of 6-OH-FAD.

As far as activity is concerned, it should be noted that this holoenzyme has a better capacity to reduce ferredoxin than artificial dyes (Table 1).

Table 1. *Catalytic activities of apoferredoxin – $NADP^+$ reductase reconstituted with FAD analogues*

FAD analogue	Midpoint potential[a]	Activity with			
		$K_3Fe(CN)_6$	iodonitrotetrazolium	cyt *c*	ferredoxin → cyt *c*
	mV	%			
FAD	−208	100	100	0	100
1-Deaza-FAD	−280	7	2	0	2
2-Thio-FAD	−126	900	130	18	20
6-OH-FAD	−265[b]	4	10	1	14
8-Cl-FAD	−152	650	515	13	15
8-Mercapto-FAD	−290	2.5	6	0.5	1
8-SCH_2COOH-FAD	−208	9	30	2	13
8-SCH_2CONH_2-FAD	–	24	61	6.5	8

[a] Midpoint potential of riboflavin level
[b] P. Hemmerich, unpublished data

Comparison of the Catalytic Competency of Ferredoxin – $NADP^+$ Reductase Reconstituted with Various FAD Analogues

The apoprotein of ferredoxin-$NADP^+$ reductase was reconstituted with different analogues of FAD and the activities with different electron acceptors tested. In Table 1 the oxidation-reduction potentials of the free FAD analogues are reported, together with the activities of the reconstituted holoenzyme.

Examining first the ferricyanide reductase activity, it can be seen that 2-thio-FAD, with a redox potential 82 mV more positive than FAD, produces a holoenzyme with 900% higher catalytic rate than FAD-reconstituted enzyme. At the other end of the oxidation-reduction scale, 8-mercapto-FAD has a redox potential 82 mV more negative than FAD and gives an enzyme with 1/40 of the rate of the FAD · enzyme. Thus, the catalytic rate of ferredoxin – $NADP^+$ reductase may be controlled by the input or output of electrons to the bound flavin. In fact, when ferricyanide is the electron acceptor, the modification of the flavoprotein redox potential obtained through the substitution of FAD with flavin analogues of different redox potential results in a variation of the rate over a 100-fold range. The semilog plot of rate versus flavin two-electron potential [19] shows that a linear relationship exists (with the exception of 8-SCH_2COOH-FAD · enzyme) and the sign of the slope implies that it is reduction of the enzyme-bound FAD (or analogue) which is rate-determining. No such simple relation with the flavin redox potential is found for iodonitrotetrazolium reductase activity.

On the other hand, the examination of the data (Table 1) obtained using the physiological one-electron carrier ferredoxin reveals a different pattern. The rate with this acceptor is about tenfold lower than with ferricyanide. There is no linear free energy relationship between redox potential and rate in the range tested. With the exception of 1-deaza-FAD, all the other FAD analogues when reconstituted with the apoprotein conferred to the enzyme the ability to reduce cytochrome *c* directly to various extents. This activity, which seems absent in the native ferredoxin – $NADP^+$ reductase, is partially inhibited by addition of superoxide dismutase, thus suggesting a mediation of superoxide radical in the cytochrome reduction. On the other hand, the inhibition by superoxide dismutase is less pronounced or even absent when reduction of cytochrome *c* is measured in the presence of ferredoxin; thus it is not clear if a direct reduction of cytochrome *c* is still taking place. In the case of the 6-OH-FAD · enzyme and of the 8-SCH_2COOH-FAD · enzyme, the difference between the two reductase activity levels (see last two columns of Table 1) leaves no doubts on the presence of a specific ferredoxin reductase activity; vice versa, the data obtained with the 2-thio-FAD, 8-Cl-FAD and 8-SCH_2CONH_2-FAD do not give a clear answer, a different assay being required to measure the ferredoxin reductase activity of these reconstituted enzymes.

Isoelectrofocusing Experiments

In order to detect whether subtle modifications were induced in the apoprotein by binding to differently substituted flavins, we analyzed the isoelectrofocusing patterns of the different reconstituted holoenzymes. The apoprotein, on binding the various FAD analogues, reformed exactly the same species as with normal FAD.

DISCUSSION

One of the more challenging problem encountered in flavoprotein biochemistry is to understand the ways in which the versatile chemistry of the isoalloxazine nucleus becomes restricted to a well defined reaction pathway by the interaction with the apoprotein moiety of a particular flavoenzyme. A further problem to be solved is to determine which way electrons from substrates enter or leave the isoalloxazine nucleus of the flavin coenzyme.

According to Massey and Hemmerich [1], ferredoxin – $NADP^+$ reductase has been assigned to class IV of flavoproteins (the dehydrogenases/electron transferases) on the basis of its reaction mechanism: it accepts electrons from a one-electron carrier (i.e. ferredoxin) and donates them to a two-electron carrier (i.e. $NADP^+$). The analysis of the results reported in the present paper clearly indicates that the properties of ferredoxin – $NADP^+$ reductase agree quite well with those of other class IV flavoproteins. The fact that the apoferredoxin – $NADP^+$ reductase binds both the thiolate form of 8-mercapto-FAD and the neutral form of the 6-OH-FAD is indicative of the lack of a positively charged group near the $-N_1-C_2=O$ position of the pyrimidine subnucleus of the flavin. This is keeping with the well known stabilization of the blue neutral semiquinone by ferredoxin – $NADP^+$ reductase and generally by flavodehydrogenases of class IV as well [1].

The ferredoxin – $NADP^+$ reductases reconstituted with various flavin analogues (8-mercapto-FAD, 2-thio-FAD, 8-SCH_2CONH_2-FAD, 6-OH-FAD) are reducible by NADPH. A relationship with the midpoint potential of the flavin analogues is apparent. Thus, the 2-thio-FAD · enzyme is reduced more rapidly than the 8-mercapto-FAD · enzyme or the 6-OH-FAD · enzyme. In addition, a linear relationship is obtained by analysis of the catalytic activity of the substituted enzymes with ferricyanide as electron acceptor. In the case of the native enzyme, the rate-limiting step in the ferricyanide reductase reaction has been shown to be the reduction of the flavin by NADPH [20]. Thus, the observed dependence of the ferricyanide reductase activity on the redox potential of the flavin probably reflects simply the greater ease of reduction the higher the redox potential of the flavin. With ferredoxin as acceptor, electron transfer between flavin and iron-sulfur

center is known to take place in a complex of the two proteins [21]. Hence, the catalytic velocity in this reaction is subject to geometrical constraints which would not apply in the case of simple molecules such as ferricyanide, e.g. to subtle differences in binding orientation or to differences in the binding constant for the flavoprotein-ferredoxin complex.

A study of the degree of accessibility to the solvent of the isoalloxazine nucleus in ferredoxin – $NADP^+$ reductase has provided interesting results. As already pointed out [3, 4], the reactivity of 8-substituted flavins and of 2-thioflavins towards specific reagents can be used to obtain information on the accessibility of the solvent to these particular positions of the protein-bound flavin. In ferredoxin – $NADP^+$ reductase we seem to have a clear-cut situation: while the 8-position of the isoalloxazine nucleus is fully reactive to methylmethanethiolsulfonate and iodoacetamide, the 2-position is completely unreactive. These results suggest that the 8-position of the flavin in ferredoxin – $NADP^+$ reductase is freely available to solvent, whereas the flavin pyrimidine subnucleus must be completely buried by amino acid residues within the protein. In regard to the enhancement of the rate of reaction of the 8-mercapto group with iodoacetamide in the 8-mercapto-FAD·enzyme compared with free 8-mercaptoflavin, it has been proposed that the enhancement could be due to a decreased polarity of the protein environment in proximity of position 8 of the flavin [3].

A final comment should be made on the isoelectrofocusing data. This technique is not able to discriminate between native enzyme and the holoenzyme reconstituted with different flavin analogues, all these forms presenting nearly identical isoelectrophoretic patterns. Such a result indicates that substitution at the isoalloxazine nucleus induces only minor, local changes without producing gross conformational modifications of the protein.

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REFERENCES

1. Massey, V. & Hemmerich, P. (1980) *Biochem. Soc. Trans. 8*, 246–257.
2. Massey, V., Ghisla, S. & Moore, E. G. (1979) *J. Biol. Chem. 254*, 9640–9650.
3. Schopfer, L. M., Massey, V. & Claiborne, A. (1981) *J. Biol. Chem. 256*, 7329–7337.
4. Claiborne, A., Massey, V., Fitzpatrick, P. F. & Schopfer, L. M. (1982) *J. Biol. Chem. 257*, 174–182.
5. Burnett, R. M., Darling, G. D., Kendall, D. S., LeQuesne, M. E., Mayhew, S. G., Smith, W. W. & Ludwig, M. L. (1974) *J. Biol. Chem. 249*, 4383–4392.
6. Hofsteenge, J., Vereijkem, J. M., Beintema, J. J., Wierenga, R. K. & Drendth, J. (1980) *Eur. J. Biochem. 113*, 141–150.
7. Thieme, R., Pai, E. F., Schirmer, R. H. & Schulz, G. E. (1981) *J. Mol. Biol. 152*, 763–782.
8. Mayhew, S. G., Whitfield, C. D., Ghisla, S. & Schuman-Jörns, M. (1974) *Eur. J. Biochem. 44*, 579–591.
9. Zanetti, G. & Curti, B. (1980) *Methods Enzymol. 69*, 250–255.
10. Karplus, P. A. & Herriot, J. R. (1982) in *Flavins and Flavoproteins* (Massay, V. & Williams, C. H., Jr, eds) pp. 28–31, Elsevier/North-Holland, New York.
11. Zanetti, G., Cidaria, D. & Curti, B. (1982) *Eur. J. Biochem. 126*, 453–458.
12. Spencer, R., Fisher, J. & Walsh, C. (1976) *Biochemistry, 15*, 1043–1053.
13. Moore, E. G., Ghisla, S. & Massey, V. (1979) *J. Biol. Chem. 254*, 8173–8178.
14. Zanetti, G. (1981) *Plant Sci. Lett. 23*, 55–61.
15. Righetti, P. G. & Drysdale, J. W. (1976) *Isoelectric Focusing*, pp. 450–463, North-Holland, Amsterdam.
16. Gozzer, C., Zanetti, G., Galliano, M., Sacchi, G. A., Minchiotti, L. & Curti, B. (1977) *Biochim. Biophys. Acta, 485*, 278–290.
17. Zanetti, G. & Curti, B. (1982) in *Flavins and Flavoproteins* (Massey, V. & Williams, C. H., Jr, eds) pp. 667–671, Elsevier/North-Holland, New York.
18. Zanetti, G. & Forti, G. (1969) *J. Biol. Chem. 244*, 4757–4760.
19. Light, D. R. & Walsh, C. (1980) *J. Biol. Chem. 255*, 4264–4277.
20. Massey, V., Matthews, R. G., Foust, G. P., Howell, L. G., Williams, C. H., Jr, Zanetti, G. & Ronchi, S. (1970) in *Pyridine Nucleotide-Dependent Dehydrogenases* (Sund, H., ed.) pp. 393–409, Springer-Verlag, New York.
21. Foust, G. P., Mayhew, S. G. & Massey, V. (1969) *J. Biol. Chem. 244*, 964–970.

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