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INVOLVEMENT OF THE OXIDIZED–SEMIQUINONE COUPLE IN PHOTOREDUCTION OF ALGAL FLAVODOXINS

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Key Word Index—*Chondrus crispus*; *Porphyra umbilicalis*; Rhodophyta; flavodoxin; redox potentials.

Abstract—A flavodoxin was isolated from the red macroalga *Porphyra umbilicalis* and the redox potentials for the oxidized–semiquinone (E_2) and semiquinone–hydroquinone (E_1) couples determined using a xanthine–xanthine oxidase reducing system in conjunction with Safranine O as a reference dye. The value of E_2 was -273 mV at pH 7.0, while that for E_1, determined by calculating the semiquinone formation constant, was -424 mV. Similar values were obtained for the flavodoxin from another red alga, *Chondrus crispus*. From these data, and knowing the association constant for the binding of FMN to the apoflavodoxin, the association constants for binding of the flavin semiquinone and hydroquinone could be calculated, and also the standard free energy changes for all the possible interactions. Since E_2 is pH dependent its redox potential at pH 8, the pH of the chloroplast stroma during light harvesting, is close to that of the $NADP^+$–NADPH couple. Thus, at pH 8 the oxidized–semiquinone couple would be largely reduced, suggesting that this couple may be functional in $NADP^+$ photoreduction rather than the semiquinone–hydroquinone couple, generally assumed to be the physiologically important interconversion. The observations are of particular significance for *Chondrus crispus* since this organism lacks ferredoxin, which sustains this role in other organisms carrying out oxygenic photosynthesis.

INTRODUCTION

Flavodoxins are low-potential electron-transfer proteins with an FMN as the redox-active component and, consequently, can apparently substitute fully for ferredoxins in the range of biological reactions supported by these in prokaryotes and some algae [1, 2]. Often, flavodoxins are produced in response to iron deficiency [3] but in a few cases they are constitutive, occurring either in addition to a ferredoxin or, very rarely, as the only low-potential electron-transfer protein in significant amount. Consequently, amongst their other properties that of redox potential is of particular significance. The reduction of a flavodoxin occurs via an intermediate, stable FMN semiquinone formed by addition of one reducing equivalent, and subsequently to the hydroquinone by a further reducing equivalent. A simple representation of this reduction can be written:

$$\underset{}{PF} \overset{e^- + H^+}{\rightleftharpoons} \underset{E_2}{PFH^\bullet} \overset{e^-}{\rightleftharpoons} \underset{E_1}{PFH^-}$$

where P represents the protein component, the apoflavodoxin, here with the bound flavin (F). Accordingly, redox potentials for both the oxidized–semiquinone (E_2) and the semiquinone–hydroquinone (E_1) couples are of interest, although it is generally assumed that it is the more reducing $PFH^\bullet$–PFH^- couple that is physiologically important. The value of E_2 is dependent on pH; that for E_1 in some flavodoxins is also pH dependent at about pH 6 and lower.

Amongst the methods used to determine the redox potential for the oxidized–semiquinone transition have been potentiometric titration using mediators to faciliate electron transfer from the electrode to the protein, and, for example, dithionite to provide reducing equivalents at low potential, or by equilibration with a mediator dye of suitable redox potential. The former has also been used to study the less amenable semiquinone–hydroquinone couple, with the alternatives including: reduction by NADPH in conjunction with ferredoxin–$NADP^+$ oxidoreductase; H_2 and hydrogenase; and photochemical reduction with 5-deaza-flavin [2, 4]. For the non-specialist laboratory there are difficulties in making most of these determinations, and a recent report [5] proposing a more convenient method applicable to flavoproteins has thus been of interest. This procedure, which exploits the xanthine–xanthine oxidase reducing system, has been used to determine the redox potentials of a flavodoxin from a red alga, *Porphyra umbilicalis*, and, based on that finding, to re-evaluate the redox potentials of the *Chondrus crispus* flavodoxin, found previously by potentiometric titration [6].

Abbreviations: K_{ox}, K_{sq} and K_{hq} represent association constants(K_a) for binding of flavin in oxidized, semiquinone and hydroquinone forms, respectively, to the apoflavodoxin. K_{SQ} is the flavodoxin semiquinone formation constant.

RESULTS AND DISCUSSION

The prerequisite of the xanthine–xanthine oxidase method for measuring the redox potential E_2 of the flavodoxin is the use of a reference dye with an E_m within about 25 mV of the anticipated value for E_2 and which has an absorption peak present in either the oxidized or reduced state that is close to an isosbestic point in the quinone to semiquinone transformation. This enables changes in reduction state of the reference dye to be followed, at the same time that flavodoxin reduction is being monitored by changes in the A_{max} of the semiquinone, where the dye is preferably non-absorbing. To evaluate E_1 an alternative system with these appropriate criteria would be needed, but instead, as in the present study, it can be found from the semiquinone formation constant K_{SQ}. This can be determined from the reduction of the flavodoxin under anaerobic conditions by the xanthine–xanthine oxidase system in the presence of a low-potential mediator(s) but in the absence of the reference dye.

$$K_{SQ} = [PFH^{\bullet}]^2/[PF][PFH^-]$$

Consideration of the values of E_2 for flavodoxins from photosynthetic prokaryotes [2, 4] and of the redox potentials of a range of dyes [7, 8] suggested the suitability of Safranine O ($E^{\circ\prime}$ − 280 mV, A_{max} 520 nm for the oxidized form) as the reference dye. A preliminary experiment following dye reduction by NADPH ($E^{\circ\prime}$ − 320 mV) gave a $E^{\circ\prime}$ for Safranine O close (10 mV) to this value, for a two-electron transfer. Safranine O has a pK on the reduced form at pH 5.8; above this the H^+/e^- stoichiometry of the redox reaction is 1.0, and the change in E_m is − 30 mV per unit of pH increase [8].

Determination of K_{SQ}

The anaerobic reduction at pH 7.0 of the oxidized flavodoxin to the blue, neutral radical semiquinone occurred over 100 min (Fig. 1). The initial lag is attributed to residual trace amounts of oxygen. Compared with the absorption spectrum of the oxidized flavodoxin in the visible region (A_{max} 380, 463, 487sh nm) the semiquinone spectrum had A_{max} at 350, 376sh and 583, 628sh nm. There were two well-defined isosbestic points in the range 320 to 750 nm, at 361 and 512 nm (Fig. 1(a)). Further reduction to the hydroquinone (Fig. 1(b)) took another 270 min, with isosbestic points at 321, 386 and 410 nm. The plot of molar absorption coefficient at 583 nm for the flavodoxin species present at each time (Fig. 2) gave a value of 4075 $M^{-1}cm^{-1}$ for the semiquinone.

The plot shows that some 90% of the theoretical semiquinone was formed in the reaction, and consequently that under the conditions of the experiment

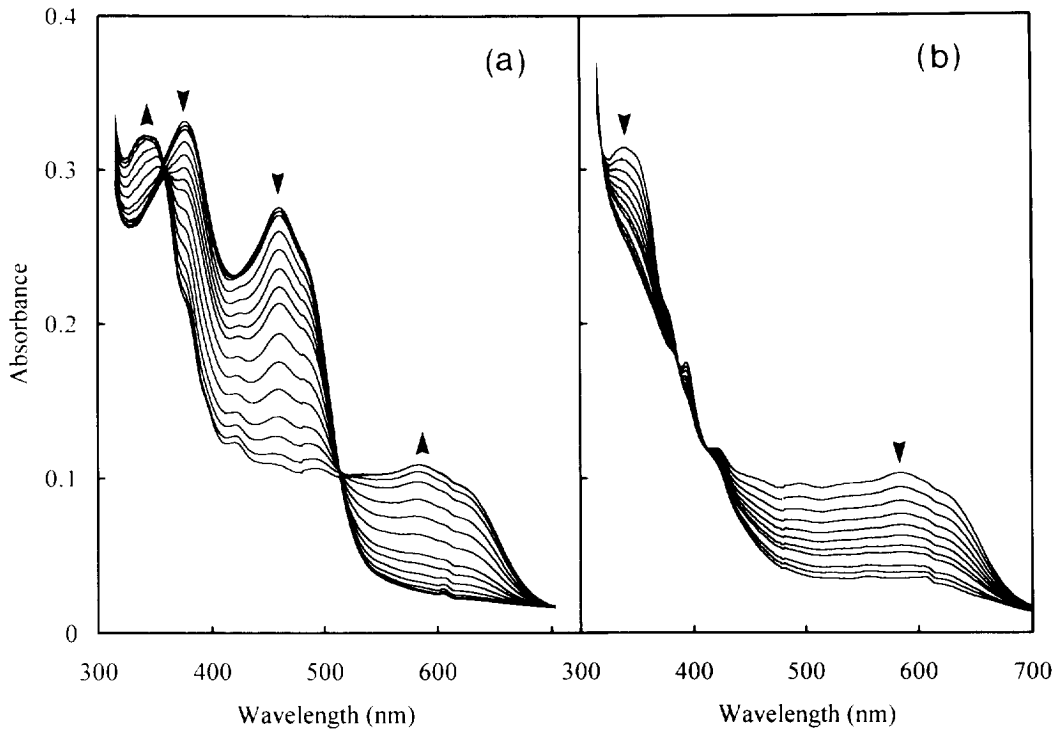

Fig. 1. Determination of K_{SQ} for *P. umbilicalis* flavodoxin. Spectra were recorded before, and following addition of xanthine oxidase to flavodoxin in the presence of xanthine and methyl- and benzyl-viologens in an anaerobic cell. The spectra were (a) during semiquinone formation at 0, 2, 5 and then 5-min intervals to 30 min, and 10-min intervals to 100 min; (b) during hydroquinone formation at 120 min and then 20-min intervals to 260 min, and then at 260, 303 and 330 min.

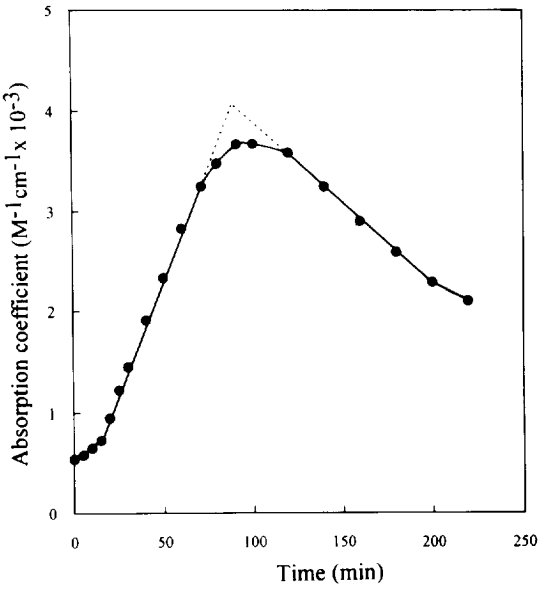

Fig. 2. Amounts of *P. umbilicalis* flavodoxin semiquinone during anaerobic reduction of the oxidized flavodoxin. The data points are derived from the spectra in Fig. 1 and are expressed as molar absorbance coefficients at 583 nm.

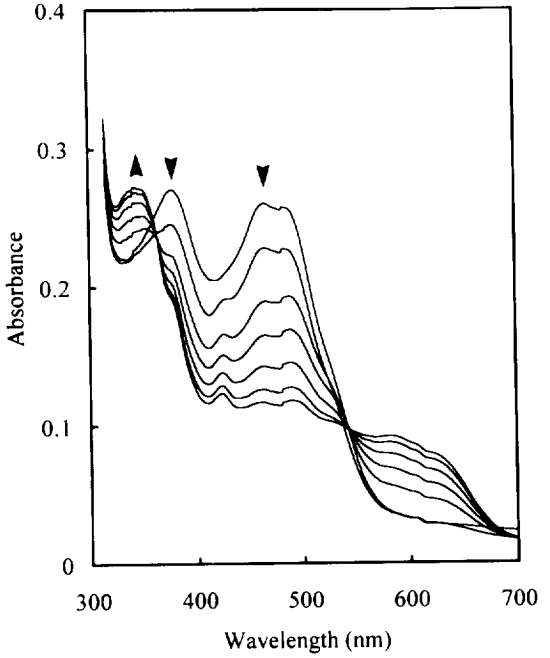

Fig. 3. Reduction of *P. umbilicalis* flavodoxin to the semiquinone in conjunction with Safranine reduction. A spectrum was recorded initially and at 10, 20, 25, 27.5, 30, 32.5 and 35 min after initiating reduction.

K_{SQ} was 326. The value of $E_2 - E_1$ was calculated as 151 mV from the relationship

$$E_2 - E_1 = (2.303\ RT/nF)\log K_{SQ}$$

where R, T, n and F have their conventional designations and appropriate units.

Determination of E_2

In the reduction with Safranine present as the reference dye the semiquinone was formed in some 35 min (Fig. 3), and the series of spectra taken over this time showed isosbestic points at 361 and 539 nm; the latter was red-shifted by 27 nm compared with flavodoxin reduction in the absence of the dye (Fig. 1(a)). For each of the spectra the oxidation state of the flavodoxin was estimated from A 583 nm and that of the Safranine from A 512 nm. Since the concentration of the flavodoxin was, within experimental error, as for the data in Figs 1 and 2, the values for absorbances corresponding to total conversion to the semiquinone and to the isosbestic point at 512 nm were known. Logarithmic plots of ratios of PF/PFH$^\bullet$ for the flavodoxin ($n = 1$) versus ox/red for the reference dye ($n = 2$) (Fig. 4) were linear with a slope of 0.5, and the ΔE for the former compared with Safranine O was 7 mV; the flavodoxin was slightly more reduced than the Safranine, and consequently E_2 was -273 mV. In conjunction with the determination of $E_2 - E_1$ as 151 mV, the value of E_1 is therefore -424 mV.

The flavodoxin semiquinone was further reduced to the hydroquinone over the following 145 min, with isosbestic points well defined at 321, 386 and 410 nm (data not shown); these spectra resembled closely those of Fig. 1(b). In principle a similar experiment to the determination of E_2, but using a reference dye with A_{max} close to one of these isosbestic points and an E_m about -400 mV, would enable E_1 to be found directly; a likely candidate is methyl viologen ($E^{\circ\prime} - 446$ mV;

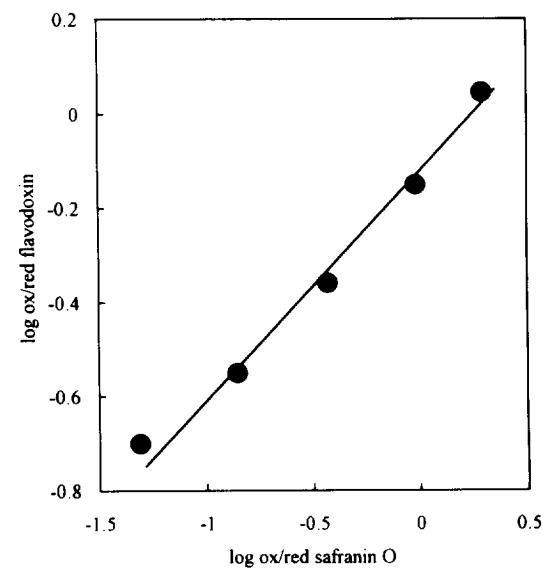

Fig. 4. Determination of E_2 for *P. umbilicalis* flavodoxin by comparison of the relative oxidation state with that of Safranine, using absorbances at 583 and 512 nm, respectively, from the spectra in Fig. 3.

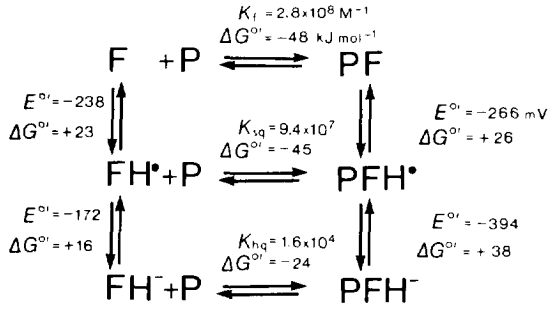

Fig. 5. K_a and $\Delta G^{\circ\prime}$ values for flavin interconversions and interactions with *P. umbilicalis* apoflavodoxin.

A_{max} 378 nm). It should be noted that these data will be for the predominant of the two flavodoxins found in *P. umbilicalis* [6], and the redox properties of the minor form may differ. The green alga *Chlorella fusca*, grown under iron deficiency, also possessed two flavodoxins; their redox potentials have recently been reported [9] but neither flavodoxin I ($E_2 = -180$ mV at pH 7.0) nor flavodoxin II ($E_2 = -218$ mV) are as electronegative as the *P. umbilicalis* flavodoxin and only the semiquinone–hydroquinone couple of flavodoxin II would support effectively photosystem 1 activity.

The values of E_2 and E_1 for *P. umbilicalis* flavodoxin were some 40–50 mV more negative than those reported for the flavodoxin from another red alga, *C. crispus* [4]. Since the potentiometric titrations of the latter had provided appreciable difficulty (Fig. 4 of ref. [4]) and the derived values of E_2 and E_1 consequently lacked some surety, these were reassessed by the present methodology. These data were closely similar to those of Figs 1–4 and gave a molar absorption coefficient for the semiquinone at 583 nm of 4050 $M^{-1}\,cm^{-1}$. The values for E_2 and E_1 were more negative than reported previously (Table 1), and are appreciably more negative than those reported for the flavodoxins from prokaryotes [4, 10].

From these revised values and the reduction potentials for free FMN [11], and using a previously determined value for the association constant for FMN binding K_f [12], the association constants for semiquinone (K_{sq}) and hydroquinone (K_{hq}) forms of FMN with the apoflavodoxin were calculated. Although the value of K_a used had been obtained from data at pH 7.7, other studies [13] have suggested that FMN binding to apoflavodoxin was independent of pH over the range 6–9. The value of K_f for *C. crispus* flavodoxin is very similar to that for other flavodoxins despite the extremely large conformational change undergone by the apoflavodoxin on flavin association or dissociation [12]. From the E_m and K_a data the free energy changes $\Delta G^{\circ\prime}$ for the various interconversions can be determined and these thermodynamic data are presented in Fig. 5, although it is not appropriate to discuss them other than briefly in the present context. In contrast with other flavodoxins studied in these respects there was no increase in binding of the flavin in going from the oxidized to the semiquinone form; as for other flavodoxins, the hydroquinone was bound much less tightly. For *C. crispus* flavodoxin K_{sq}/K_f is about 0.33, and K_{sq}/K_{hq} is *ca* 6000. These data can be compared with those for three other flavodoxins; for that from *Anabaena* 7120 the corresponding ratios were 5 and 2000 [10] and for *Anabaena* 7119 flavodoxin were 4 and 5000 [the latter is calculated from an E_1^{FMN} taken as -172 mV], and is not the figure for K_{sq}/K_{hq} given in ref [14]; while for *Megasphaera elsdenii*, K_{sq}/K_f was 120 and K_{sq}/K_{hq} was 2500 (from K_a values quoted in ref [13]). The ratios as presented for *Azotobacter vinelandii* flavodoxin [15] are now on less sure ground given the later finding that this organism contains three flavodoxins [16]. The decreased affinity for the hydroquinone has been attributed to electrostatic repulsion due to the N(1)–O(2) centre of the reduced flavin.

The redox properties of the flavodoxins from prokaryotes generally have suggested that the oxidized flavin is not involved in the electron-carrying function, but that it is semiquinone–hydroquinone interconversion that is physiologically important. This has been demonstrated in the case of flavodoxins in several nitrogen-fixing organisms [17]. In flavodoxin-containing organisms carrying out oxygenic photosynthesis there is the dilemma of the oxygen-sensitive flavodoxin semiquinone–hydroquinone couple functioning in an environment where oxygen is being produced. The observation, therefore, that the E_2 values for the red-algal flavodoxins are significantly more negative than those of

Table 1. Redox potentials at pH 7.0 of flavodoxins from red algae

	Oxidized–semiquinone E_2 (mV)	Semiquinone–hydroquinone E_1 (mV)
C. crispus	−261*	−394
	−271	—
P. umbilicalis	−273	−424
	−294	—

*Determined at pH 8.0 and corrected to pH 7.0. Values of E_2 and E_1 for *C. crispus* flavodoxin from potentiometric data given in ref. [4] were −235 mV and −370 mV, respectively. Data for E_2 are from experiments on separate occasions.

other flavodoxins, although values for E_1 are similar [2, 4], deserves attention. One consequence is that the difference between E_2 and E_1 for the algal flavodoxins is decreased, and at pH 8, which is favourable for CO_2 assimilation, they differ by only some 80 mV. Perhaps more significant, however, is the observation that pH 8.0 the redox potential for the less oxygen-sensitive oxidized–semiquinone couple (− 330 mV) would be close to that for the $NADP^+$–NADPH couple (− 350 mV). In higher plants, either in *Spinacia oleracea* (spinach) chloroplasts [18] or *Pisum sativum* (pea) leaves [19], under conditions of good illumination and availability of CO_2, the NADP(H) can be up to about 75% reduced. This corresponds to an E_h of − 360 mV for the $NADP^+$–NADPH couple. Even under conditions of darkness, probably about 40–60% NADPH is present, giving an E_h of, maximally, − 315 mV. An E_2 value of − 330 mV for the oxidized–semiquinone couple at pH 8 suggests some 80% of the flavodoxin would be in the semiquinone form if this couple were operative in $NADP^+$ photoreduction, whereas if the more negative E_1 of the semiquinone–hydroquinone couple were used, only some 20% would be present as the hydroquinone. Coincidentally, for either couple the same amount of semiquinone would be present. In *P. umbilicalis* it is difficult to envisage a specific role for the flavodoxin since a ferredoxin of E_m − 380 mV is present and this couples effectively to $NADP^+$ photoreduction [20]. However, *C. crispus* lacks a ferredoxin and here the probability that it is the flavodoxin oxidized–semiquinone couple that is the physiologically important transition in $NADP^+$ photoreduction, rather than as generally assumed the semiquinone–hydroquinone couple, is of considerable interest.

EXPERIMENTAL

Isolation of flavodoxins. Flavodoxins were isolated by established methods from *C. crispus* [1, 21] and *P. umbilicalis* [1, 6], but using chromatography on DEAE-Sephadex A25 (18 cm × 2.5 cm column) rather than on DEAE-cellulose (30 cm × 2.5 cm column). The yield was *ca* 10 mg flavodoxin from 6 kg wet wt of *C. crispus*, and the protein was homogeneous on non-denaturing PAGE (20%w/v acrylamide); the *A* 464 nm/*A* 274 nm ratio was 0.21. For *P. umbilicalis* the yield was somewhat less and, while homogeneous with respect to staining on 12–20% (w/v) linear gradient acrylamide gels with Coomassie Brilliant Blue, minor impurities were detectable on silver staining. This may have been the cause of a somewhat lower *A* 463 nm/*A* 274 nm ratio of 0.17. A small amount of a second flavodoxin (R_m 0.89 cf. 0.90 on PAGE) was also detectable; the origin of this isoform remains uncertain [22].

Determination of K_{SQ}. Fully oxidized flavodoxin at 30 μM (ε 464 nm = 10 700 M^{-1} cm^{-1}) in 0.15 M Tris–HCl, at a pH in the range 6–8, containing 200 μM xanthine and 20 μM methyl viologen ($E^{\circ\prime}$ − 449 mV) was added to the chamber of a Thunberg cuvette of light-path 1 cm fitted with a side-tube to permit evacuation and purging with O_2-free N_2 via a 3-way valve. The side-tube contained sufficient xanthine oxidase ($E^{\circ\prime}$ − 90 mV) such that on addition to the cuvette chamber by tipping, its concn was 105 nM (*P. umbilicalis* studies) or 310 nM (*C. crispus* studies), and the final reaction vol. was 3 ml. The visible spectrum over the range 320–750 nm was recorded initially and at intervals of a few min until the flavodoxin was reduced to the semiquinone and then the hydroquinone form. All experiments were at 25°. Interconversion of data within the range pH 6–8 assumed that, as shown previously [4] and as for *Anabaena* 7120 flavodoxin [10], there was no dependence of E_1 on pH, and that E_2 showed a $\Delta E/\Delta$pH of − 59 mV.

Determination of E_2. The procedure was as for the determination of K_{SQ} except that the cuvette additionally contained 3 μM Safranine O, and that xanthine oxidase was 310 nM in all cases. Spectra were recorded from immediately prior to addition of xanthine oxidase to initiate flavodoxin reduction to the completion of hydroquinone formation.

Safranine O, xanthine and xanthine oxidase were from Sigma, and methyl viologen from BDH, U.K.

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