Light-induced changes of NADPH fluorescence in isolated chloroplasts: a spectral and fluorescence lifetime study¹

Gwendal Latouche *, Zoran G. Cerovic, Fabrice Montagnini, Ismael Moya

Laboratoire pour l’Utilisation du Rayonnement Electromagnétique, Université de Paris XI, 91898 Orsay, France

Received 30 March 2000; received in revised form 4 July 2000; accepted 10 July 2000

Abstract

Isolated chloroplasts show a light-induced reversible increase in blue-green fluorescence (BGF), which is only dependent on NADPH changes. In the present communication, we report a time-resolved and spectral analysis of this BGF in reconstituted chloroplasts and intact isolated chloroplasts, in the dark and under actinic illumination. From these measurements we deduced the contribution of the different forms of NADPH (free and bound to proteins) to the light-induced variation of BGF and conclude that this variation is due only to the redox change of the NADP pool. A simple model estimating the distribution of NADPH between the free and bound form was designed, that explains the differences measured for the BGF of reconstituted chloroplasts and intact chloroplasts. From the decay-associated spectra of the chloroplast BGF, we also deduced the participation of flavins to the green peak of chloroplast fluorescence emission spectrum, and the existence of excitation energy transfer from proteins to bound NADPH in chloroplasts. In addition, we re-examined the use of chloroplast BGF as a quantitative measure of NADPH concentration, and confirmed that chloroplast BGF can be used for non-destructive, continuous and probably quantitative monitoring of light-induced changes in NADP redox state. © 2000 Elsevier Science B.V. All rights reserved.

Keywords: Blue-green fluorescence; Decay-associated spectra; Excitation energy transfer; Flavin; Intact chloroplast; Reconstituted chloroplast; Pyridine nucleotide

1. Introduction

The first investigations on NAD(P)H fluorescence in vivo were made around 1957–1960 on bacteria and photosynthetic algae [1–4], and though studies on the potential and characteristics of this signal were continued in biotechnology (e.g., [5,6]) and in the biomedical field (e.g., [7–9]), NAD(P)H fluorescence of green plants was neglected for a long time. The progress in UV-lasers and their use for remote sensing of plants (for a review see [10]) promoted studies on UV-excited fluorescing compounds of leaves, among which NADPH is of particular importance because it links the reducing side of Photosystem I to carbon...
metabolism. There are many other blue-green fluorophores in the leaf, mostly belonging to the phenylpropanoid family. The latter compounds are located in the cell walls and vacuoles, or in the cuticle of the epidermis. The leaf content of these fluorophores is dependent of plant type and environmental conditions [10], but they always represent the major part of the blue-green fluorescence (BGF) of leaves. They also absorb UV-radiation before it reaches the chloroplasts and therefore make a screen to fluorophores located in the chloroplasts. In addition, blue-green chloroplast fluorescence is inherently low (it was even said to be negligible [11,12]) therefore the contribution of chloroplasts to the BGF of leaves is often very small. It was estimated to be around 5% in spinach [13] and 10–15% in pea [14]. Thus, in most cases NADPH fluorescence is too small to be detected on leaves [12,14,15], with the exception of young pea leaves grown without UV light [14]. By contrast, research on the NADPH fluorescence of isolated chloroplasts is possible [13–15], but even then, there is a problem because chloroplast BGF is not due only to NADPH. Other potential fluorophores like pteridines, folates, pyridoxines, quinones and kinurenines [16], which are known to be present in chloroplasts, can contribute to a part of the chloroplast BGF. NAD is also present in the chloroplast but it always remains almost entirely in its oxidized form NAD$^+$ [17,18]. This form does not contribute to BGF because only the reduced form of pyridine nucleotides fluoresces when excited with UV-A radiation. A contribution of flavins and flavoproteins, which are green-emitting fluorophores [16,19] should also be considered when analyzing the chloroplast BGF. Another important phenomenon that influences the BGF of chloroplasts and leaves, by decreasing the intensity of BGF and distorting its emission spectrum, is the reabsorption of this fluorescence by photosynthetic pigments (chlorophylls and carotenoids) (cf. [14] and references therein).

It was argued that the use of BGF for quantitative measurement of light-induced changes of NADPH redox state would be best performed in a front-face configuration [13]. Indeed, front-face fluorimetry is well adapted to concentrated chloroplast suspensions. Also with front-face fluorimetry, there is a linear relation between fluorescence and fluorophore concentration when all the fluorescence-exciting light is absorbed within the sample and when the absorbance of the fluorophore is negligible compared with the total absorbance [20], which is the case for concentrated chloroplast solutions, and provided that there is no self-reabsorption (inner-filter effect) [21], a phenomenon that occurs only for high concentrations of fluorophore.

Isolated chloroplasts show a reversible increase of BGF when illuminated with actinic light, and it has been proposed that this light-induced variation of chloroplast BGF is completely NADPH dependent [13,14]. This opened the way for non-destructive and continuous monitoring of light-induced changes of NADP redox state in chloroplasts. Still, the question was raised about the contribution of NADPH binding to protein to the overall light-induced variable chloroplast BGF. Indeed, in chloroplasts, an important part of NADP is bound to proteins [22,23]. This binding modifies the characteristics of NADPH fluorescence, notably by increasing its fluorescence quantum yield and fluorescence lifetime. When bound to enzymes, NAD(P)H is 3–5 times more fluorescent than the free form [24–26] (larger enhancements have also been found [27]). This increase can be even more important in the case of ternary complexes, NAD(P)H–enzyme–substrate [25,28,29]. Therefore, a simple binding of NADPH to proteins can lead to an increase in the BGF signal without a change in the redox state of NADP. In addition, the contribution of flavins to light-induced variable chloroplast BGF has been averted on the basis of kinetic and time-resolved measurements [14], but full spectral analysis of variable BGF has not yet been performed.

In the present study we report a time-resolved analysis of chloroplast fluorescence at the maximum excitation and emission wavelength of NADPH, and decay-associated spectra (DAS) of chloroplast fluorescence obtained in the dark and under light that enabled us to determine the involvement of NADPH binding to proteins and the contribution of flavins to the light-induced changes of BGF. The fluorescence of two types of isolated chloroplasts, reconstituted chloroplasts and intact isolated chloroplasts, were analyzed and compared using a model which predicts the distribution of free and bound NADPH in the chloroplast. The basis for the use of NADPH fluorescence in the chloroplast, as a quantitative
measure of its concentration, was also addressed in our study.

2. Materials and methods

2.1. Plant material and chemicals

Pea (*Pisum sativum*, var. Petit provençal) was grown in a growth cabinet at 21°C day, 16°C night, a PPFD of 350 μmol photons m⁻² s⁻¹, 80% relative humidity, and a photoperiod of 16 h light, 8 h dark. Young pea shoots (8–10 days) were used in the preparations of isolated chloroplasts.

Purified ferredoxin-NADP⁺ reductase (FNR, EC 1.6.99.4) from spinach (*Spinacia oleracea*) was kindly provided by Dr Myroslawa Miginiac-Maslow, and purified D-ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco, EC 4.1.1.39) from *Lolium perrenne*, by Dr Martin Parry.

L-NADPH was purchased from Sigma (St. Quentin Fallavier, France), and 1,4-dithiothreitol was purchased from Fluka (St Quentin Fallavier, France). The other reagents were of proanalysis grade.

2.2. Chloroplast preparations

Intact isolated chloroplasts were prepared as in [30]. The preparations were 80–95% intact as judged by ferricyanide penetration [31]. Chlorophyll (Chl) concentration was determined according to [32]. A medium containing 330 mM sorbitol, 10 mM KCl, 1 mM EDTA and 50 mM Hepes buffer, adjusted to pH 7.9 with KOH, prepared as double strength, was the basis of all other media used. The isolation medium was the basic medium diluted 25-fold with 330 mM sorbitol. The resuspending medium was the basic medium with the addition of 1 mM MnCl₂, 1 mM MgCl₂ and 0.2 mM KH₂PO₄.

Samples for fluorescence measurements on intact isolated chloroplasts were obtained by adding intact isolated chloroplasts (50 μg Chl/ml final concentration) to a solution of basic medium containing 10 mM KHCO₃ and 0.2 mM KH₂PO₄.

Chloroplast extracts were obtained as in [33] with modifications. Three of the four pellets of intact chloroplast were resuspended in 1 ml of an hypotonic solution (25-fold diluted basic medium) containing 5 mM MgCl₂ and 3 mM 1,4-dithiothreitol.

After approximately 1 min (to allow the rupture of the chloroplast envelope) the suspension was centrifuged for 10 min at 13000×g. The supernatant, chloroplast extract free from Chl, was retained and kept at −80°C. The concentration of chloroplast extract was expressed as the equivalent concentration of Chl and was calculated from the total volume of chloroplast extract and the total Chl content in the precipitate.

Reconstituted chloroplasts were prepared in the measuring cell by mixing thylakoids (37 μg Chl/ml, final concentration) and ten times more chloroplast extract (the equivalent of 370 μg Chl/ml) in a solution of basic medium containing 5 mM MgCl₂, 10 mM KHCO₃, 4 mM KH₂PO₄ and 1 mM 1,4-dithiothreitol. Broken chloroplasts (type D chloroplasts [34]) were used as a source of thylakoids. They were obtained by freezing intact isolated chloroplasts at −80°C immediately after isolation, and by thawing them just before the experiments. The complete breaking of their envelope was confirmed by ferricyanide penetration.

Thylakoids fragments (type F chloroplasts [34]) were prepared from the supernatant of the second centrifugation of intact chloroplast preparation. The supernatant was centrifuged for 4 min at 4100×g. The new pellet was resuspended in 40 ml basic medium after the rupture of the chloroplast envelope by osmotic shock, then this solution was centrifuged for 10 min at 4100×g. The pellet was resuspended in the resuspension medium used for intact chloroplasts. Before use, the thylakoids fragments were heated at 100°C for 10 min, and homogenized by sonication (5–6 bursts on position no. 3 of the VC250 sonicator, Sonics and Materials, Danbury, CT, USA).

2.3. Fluorescence measurements

Fluorescence lifetime and excitation or emission DAS were measured by time-correlated single-photon-counting on the FLU3 set-up (SA4 beam-line) of the Super-ACO synchrotron in Orsay (France), as described previously [35,36], with some modifications. A double-grating monochromator (H10 D UV, Jobin-Yvon, Longjumeau, France) was used with 2 mm slits (8 nm band pass) for the excitation part.
of the apparatus. A new multichannel analyzer was used, that was an acquisition interface board (Accuspec Nai, Canberra Electronique, Savigny-le-Temple, France) with supporting software. Decay histograms were acquired until a total of 1 million counts were accumulated. Seventy-eight nanoseconds were covered by 2048 channels at 0.038 ns per channel. Deconvolution of the experimental decays into a sum of exponential was achieved by iterative convolution as previously described [14,35] using a proprietary least-square fit program based on Marquardt search algorithm for non-linear parameters [37]. The quality of the fits was judged by the reduced $\chi^2$ criterion and the plot of the weighted residual.

Standard quartz cells (101-QS, Hellma, Paris, France) with an optical path of 1 cm could be used throughout, thanks to a modification of the set-up that enabled measurements on that kind of cell in a front-face configuration. No attenuation of the measuring beam was needed for the ‘dark’ measurements. The measurements taken in the dark were fluorescence measurements except a weak measuring beam was used that did not provide enough light energy to activate photosynthesis. A laser diode (656 nm, Philips, Eindhoven, Netherlands) was added to the set-up to provide actinic photosynthetic light (PPFD of 50 $\mu$mol photons m$^{-2}$ s$^{-1}$) that did not interfere with fluorescence measurements because the emission monochromator was protected by optical filters: a blue-green glass filter (CS 4-96, Corning, ARIES, Chatillon, France) for emission spectra measurements, or a wide-band interference filter (450WB80, 450 nm, $T=80\%$, FWHM = 72 nm, Omega, Brattleboro, USA) for measurements of excitation spectra and fixed wavelength decays (emission wavelength 456 nm). For measurements with an excitation/emission wavelength of 450/550 nm, the blue-green glass filter (CS 4-96) was used in combination with a long-pass filter (KV 500, Schott, Clichy, France). The used PPFD was sufficient to achieve a maximum light-induced variation of chloroplast BGF.

Decay-associated spectra were obtained by varying the excitation or emission wavelength at which the fluorescence decays were recorded. Individual analysis was performed for each decay and then a global fit was performed simultaneously on all decays in a spectrum. The excitation spectra were corrected online for source variation by using a part of the beam deviated towards a cell of rhodamine B (cf. [38]) whose fluorescence was continuously recorded. Emission spectra were corrected by using quinine sulfate as a standard of known emission characteristics (cf. [38]).

Measurements of BGF yield were performed on a new version of the pulse-modulated fluorimeter described in [13]. A high-power xenon flash lamp (L4633, Hamamatsu, Massy, France) was used as the pulsed excitation light source (1 $\mu$s duration) and the BGF was measured with a photomultiplier (R5600U-01, Hamamatsu, Massy, France)-based detector insensitive to continuous light. The excitation wavelength was defined by a 340 nm interference filter ($T=33\%$, FWHM = 10 nm, 03FIU008, Melles Griot, Magny les Hameaux, France) and the BGF detector was protected by a UV-blocking filter (KV408, Schott, Clichy, France) and a blue glass filter (CS 4-96, Corning, ARIES, Chatillon, France). The same standard quartz cells as for the others fluorescence measurements were used, again in a front-face configuration.

All measurements were made at a controlled temperature of 20°C.

3. Results

3.1. Light-induced changes of blue fluorescence for reconstituted chloroplasts and intact chloroplasts

When excited with a non-actinic measuring UV beam, chloroplast suspensions fluoresce in the blue-green region. This ‘dark’ BGF comes from several different fluorophores, most of them are not affected by actinic light. In addition, there may be a low non-specific fluorescence of the set-up itself. So this ‘dark’ fluorescence remains unchanged in the presence of actinic light, and therefore the BGF of chloroplasts under light is the sum of this ‘dark’ fluorescence and a light-induced BGF coming from NADPH. Hence, to perform a time-resolved analysis of the light-induced variations of chloroplast BGF, it was necessary to find conditions under which the light-induced BGF is substantial compared with ‘dark’ fluorescence. In Fig. 1 the BGF of intact isolated chloroplasts in solution was recorded during a dark and a
light period showing that the light-induced variation represent only about one-third of the BGF under light. This appeared to be too small for an accurate estimation of the light-induced modifications of the characteristics of the lifetime components. As our attempts to increase the light-induced variable BGF in intact chloroplasts showed only very limited success, we tried reconstituted chloroplast systems. Reconstituted chloroplasts were very similar to intact chloroplasts except they exhibited a light-induced variation of BGF that represented about one half of their BGF under light (Fig. 1). This comes from an increased proportion of NADP in reconstituted chloroplasts. Given the proportion of chloroplast extract and of thylakoids used for reconstituted chloroplasts, the final stroma/thylakoid ratio was 11 times greater in reconstituted chloroplasts than in intact chloroplasts. Thus, for the same Chl concentration there was more light-induced BGF, because there was more NADP.

Although the parent intact chloroplasts showed a relatively good O$_2$ evolution rate (around 100 μmol O$_2$ (mg Chl)$^{-1}$ h$^{-1}$), the reconstituted chloroplasts had a low rate of photosynthetic electron transport under the conditions used for fluorescence measurements (below 10 μmol O$_2$ (mg Chl)$^{-1}$ h$^{-1}$). This was due to the dilution of stromal compounds, and we did not attempt to remedy to it, because low rates were beneficial for the long lasting fluorescence measurements.

3.2. Fluorescence decays of reconstituted chloroplasts

Several lifetime components were necessary to properly describe the fluorescence decays of reconstituted chloroplasts. With the iterative convolution technique that we used (see Section 2), the number of components is gradually increased until the $\chi^2$ value remains stable and reproducible re-convolutions are obtained. We found that five lifetime components were necessary in the case of reconstituted chloroplasts, but the use of only four components gave also very good results, and it was difficult to decide between the two. In order to verify the presence of five lifetime components by an independent method, we performed an analysis with the maximum entropy method [39] on part of the decays (decays in the dark with an excitation wavelength between 340 and 350 nm and an emission wavelength of 456 nm). In this method, the number of lifetime components is a free parameter. The resulting five fluorescence lifetimes were slightly different from those obtained by our least-square fit program, and the relative pre-exponential factors were almost the same. Given the complexity of the BGF of chloroplasts, it is reasonable to assume that all five lifetime components contain contributions from different fluorophores. Each lifetime component therefore represents a class of fluorophores having similar lifetimes, and it should be kept in mind that it is not possible to assign a lifetime component to a particular fluorophore. However, when the fluorescence lifetime(s) of a fluorophore is known, we can say to which lifetime component(s) it participates. Thus, the five resolved lifetime components can be treated accordingly, for a comparative purpose, by analyzing the variation of respective relative contribution in different conditions or samples.

To be able to compare the time-resolved analysis of chloroplast BGF in the dark and under light, we first had to check the variation of the fluorescence lifetime for each component. There were small differences in the lifetime of the components between dark and light, especially for the very short component that was the most sensitive. Still, the differences in
component lifetimes between two consecutive decays, one in the dark and the other under light, have a random direction of variation when comparing the different couples of decays, and were smaller than the variations existing between two identical samples in the dark. Therefore, we could consider that the component lifetimes are the same in the dark and under light, and we could analyze globally, with the same lifetimes, the decays measured in the dark and under light.

In Fig. 2 the distribution between the five lifetime components of the light-induced variation of reconstituted chloroplast BGF is presented. It can be seen that the four shorter components (C1–C4) are all implied in the light-induced variation, whereas the very long component (C5) is not. NADPH fluorescence, which is responsible for the light-induced variation of BGF, must therefore be present in components C1, C2, C3 and C4. Indeed, it is well known that both free and bound NAD(P)H have several lifetime components (e.g., [40]). The time-resolved analysis of free NADPH fluorescence obtained from the present set up is presented in Table 1. It is very similar to the ones previously obtained [41–44]. This shows clearly that the free form of NADPH participates to the very short (C1) and short (C2) components. The binding of NAD(P)H to proteins increases its fluorescence lifetime [25, 40, 45], but the exact value of the lifetime depends on the protein and on the presence of a third ligand (e.g., [46, 47]). The data in the literature showed that there are essentially two fluorescence lifetimes for NAD(P)H.

Table 1

<table>
<thead>
<tr>
<th>Lifetime components</th>
<th>Mean lifetime ( \tau_m ) (ns)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Very short C1 ( \tau ) (ns) ( f ) (%)</td>
<td>( \frac{1}{\sum_{i=1}^{n} \alpha_i \tau_i} )</td>
</tr>
<tr>
<td>Short C2 ( \tau ) (ns) ( f ) (%)</td>
<td>( \frac{1}{\sum_{i=1}^{n} \alpha_i \tau_i} )</td>
</tr>
<tr>
<td>Medium C3 ( \tau ) (ns) ( f ) (%)</td>
<td>( \frac{1}{\sum_{i=1}^{n} \alpha_i \tau_i} )</td>
</tr>
<tr>
<td>Long C4 ( \tau ) (ns) ( f ) (%)</td>
<td>( \frac{1}{\sum_{i=1}^{n} \alpha_i \tau_i} )</td>
</tr>
<tr>
<td>Very long C5 ( \tau ) (ns) ( f ) (%)</td>
<td>( \frac{1}{\sum_{i=1}^{n} \alpha_i \tau_i} )</td>
</tr>
</tbody>
</table>

NADPH

<table>
<thead>
<tr>
<th>( \tau ) (ns)</th>
<th>( f ) (%)</th>
<th>( \tau ) (ns)</th>
<th>( f ) (%)</th>
<th>( \tau ) (ns)</th>
<th>( f ) (%)</th>
<th>( \tau ) (ns)</th>
<th>( f ) (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.15</td>
<td>43.1</td>
<td>0.53</td>
<td>54.3</td>
<td>2.9</td>
<td>2.6</td>
<td>–</td>
<td>–</td>
</tr>
</tbody>
</table>
| Reconstituted chloroplast
| 0.16       | 0.52 | 1.3  | 3.8  | 11   |      |      |      |

Dark

<table>
<thead>
<tr>
<th>( \tau ) (ns)</th>
<th>( f ) (%)</th>
<th>( \tau ) (ns)</th>
<th>( f ) (%)</th>
<th>( \tau ) (ns)</th>
<th>( f ) (%)</th>
<th>( \tau ) (ns)</th>
<th>( f ) (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>9.7</td>
<td>7.9</td>
<td>18.6</td>
<td>31.2</td>
<td>25.3</td>
<td>15.2</td>
<td>3.15</td>
<td></td>
</tr>
</tbody>
</table>
| Light
| 12.1           | 22.5 | 31.9 | 24.7 | 8.8  | 2.46 |      |      |

The data represent the mean values and standard deviations (in brackets) of nine fluorescence decays. These data were obtained by global analysis of nine fluorescence decays for NADPH and 18 fluorescence decays (nine in the dark and nine under light) for reconstituted chloroplasts. A solution of NADPH (10 \( \mu \)M) in the basic medium was used for the time-resolved fluorescence measurements of the pure cofactor. The emission wavelength was 456 nm for all measurements. The excitation wavelength was 340 nm for NADPH and between 340 and 350 nm for reconstituted chloroplasts.

Fractional intensities were defined as: \( f_i = \alpha_i \tau_i / \sum_{i=1}^{n} \alpha_i \tau_i \) and the mean lifetime as: \( \tau_m = \sum_{i=1}^{n} f_i \tau_i = \sum_{i=1}^{n} \alpha_i \tau_i / \sum_{i=1}^{n} \alpha_i \tau_i \) where \( \alpha_i \) represent the relative pre-exponential factors of the decay function (\( \sum_{i=1}^{n} \alpha_i = 1 \)).
bound to proteins: one around 1–2 ns and another around 3–6 ns [29,44,47–49]. Thus, the bound form of NADPH will participate to the medium (C3) and long (C4) components. We tried to measure the life-time of NADPH bound to FNR and Rubisco, which are the most abundant proteins in the chloroplast known to bind NADPH. The binding of NADPH to Rubisco, which may appear strange at first sight, is non-specific and has been described by Badger and Lorimer [50]. Unfortunately, our attempts to measure NADPH fluorescence lifetimes in these two complexes were unsuccessful.

There is no doubt that the photosynthetic reduction of NADP$^+$ to NADPH under light plays an important role in the light-induced increase of BGF in chloroplasts. But, because of the increase of NADPH fluorescence yield once bound to proteins, a light-induced increase in the ratio (bound NADPH)/free NADPH) could also contribute to the light-induced increase in chloroplast BGF. If this were the case, we should observe an increase in the mean fluorescence lifetime under light and especially an increase of the fractional intensities of the component C3 and C4 to the expense of the component C1 and C2. Still, as can be seen in Table 1, there were no significant variations of the fractional intensities of the first four components. The mean lifetime was decreased under light as a consequence of the larger contribution of the first four components compared with the C5 component. We can conclude that there is no significant light-induced variation in the proportion of bound NADPH in reconstituted chloroplasts, and, therefore, that the light-induced changes of BGF in reconstituted chloroplasts is totally due to the photosynthetic reduction of NADP.

### 3.3. Decay-associated excitation spectra of reconstituted chloroplasts

The excitation DAS of the BGF of reconstituted chloroplasts in the dark and under illumination are presented in Fig. 3. The total excitation spectrum has a large peak at 286 nm, that corresponds to the fluorescence excitation maximum of proteins [16]. There is a very large amount of proteins in chloroplasts (about 15 mg protein per mg Chl [51]), so the contribution of the tail of protein fluorescence emission at 456 nm is still important and can explain this peak. Furthermore, Fig. 3 shows that components C3 and C4 are the main contributors to the protein peak at 286 nm. This is in agreement with the time-resolved analysis of the fluorescence of reconstituted chloroplasts excited at 290 nm (Table 2). At this wavelength a very good time-resolved analysis of reconstituted chloroplast BGF was obtained with three components only, and the three lifetimes are close to those obtained with purified Rubisco and FNR. Still, the three components were not exactly the same as those of purified proteins, possibly because another fluorophore (or several fluorophores) has a small but

---

**Table 2**

Characteristics of the time-resolved fluorescence of Rubisco, FNR and reconstituted chloroplasts excited in the protein absorption band

<table>
<thead>
<tr>
<th></th>
<th>Excitation wavelength (nm)</th>
<th>Emission wavelength (nm)</th>
<th>Lifetime components</th>
<th>Mean lifetime $\tau_m$ (ns)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Short C'1 $\tau$ (ns) $f$ (%)</td>
<td>Medium C'2 $\tau$ (ns) $f$ (%)</td>
</tr>
<tr>
<td>Reconstituted chloroplasts</td>
<td>275–295</td>
<td>456</td>
<td>0.56 (1.2) 6.2 (1.2)</td>
<td>2.6 (2.2) 33.3 (2.2)</td>
</tr>
<tr>
<td>Rubisco</td>
<td>280</td>
<td>340</td>
<td>0.49 (0.4) 13.2 (0.4)</td>
<td>2.0 (0.5) 44.5 (0.5)</td>
</tr>
<tr>
<td>FNR</td>
<td>280</td>
<td>340</td>
<td>0.46 (0.4) 12.2 (0.4)</td>
<td>2.1 (0.5) 64.3 (0.5)</td>
</tr>
<tr>
<td>FNR</td>
<td>450</td>
<td>535</td>
<td>– –</td>
<td>1.4 (0.2) 11.9 (0.2)</td>
</tr>
</tbody>
</table>

The data represent mean values and standard deviations (in brackets, when available) of 10 (reconstituted chloroplasts) or six (FNR) fluorescence decays. These data were obtained by global analysis for each type of fluorescence decay. A solution of Rubisco (58 $\mu$M active site) in the basic medium was used. FNR was also in the basic medium at a concentration of 12 $\mu$M for 280 nm excitation and at a concentration of 20 $\mu$M for 450 nm excitation.
significant contribution to this peak (e.g., NADPH, see below).

The lifetimes of the five components in Fig. 3 are slightly different from those obtained in fixed wavelength decays (Table 1). This is due to the use of global analysis of all the decays at different excitation wavelengths to obtain this excitation DAS. Fluorescence lifetimes are not exactly the same at all excitation wavelengths because of the participation of different fluorophores, especially the proteins at the shorter wavelengths.

In Fig. 4, the excitation DAS of the light-induced variation of BGF in reconstituted chloroplasts was obtained by subtracting the DAS in the dark from the DAS under light. The difference spectrum of total fluorescence shows two peaks, one at 340 nm as expected, corresponding to the excitation maximum of NADPH, and one at 286 nm. The peak at 286 nm can be explained by a transfer of energy from proteins to NADPH. The different aspects of this energy transfer are discussed later (cf. Section 4). But we can say already that it confirms that the fluorescence of bound NADPH participates to the lifetime components C3 and C4. The very long component (C5) did not show any significant participation to the light-induced variation of chloroplast BGF on the whole spectra, confirming that NADPH does not participate to the C5 component. There is absolutely no participation of the C1 and C2 component to the

Fig. 3. Excitation DAS of reconstituted chloroplasts in the dark and under light. The actinic light PPFD was 50 μmol photon m⁻² s⁻¹. The emission wavelength was 456 nm. For details see Section 2.

Fig. 4. Decay-associated difference excitation spectra of the light-induced variation of fluorescence of reconstituted chloroplasts. It was obtained from the excitation DAS presented in Fig. 3 by subtracting the spectrum in the dark from the spectrum under light for each lifetime component.
286 nm peak, which is formed only by the C3 and C4 component. Yet, a contribution with a maximum around 260 nm could have been expected for the two shortest components (C1 and C2), as free NADPH fluorescence appears in these components, and free NAD(P)H in aqueous solution has an excitation maximum at 260 nm [19,52]. This excitation maximum, which corresponds to the absorption of the adenine moiety of NAD(P), is lost when NAD(P)H is bound to proteins in its open form [19,53]. Only a slight increase can be observed around 260 nm for the C2 component in Fig. 4. We checked that pure NADPH dissolved in the basic medium exhibits a 260 nm excitation peak with about the same height than the 340 nm peak (data not shown). In fact, the absence of the 260 nm peak is not really surprising because in suspensions of chloroplasts numerous compounds absorb at 260 nm (especially proteins that represent about 60% of intact chloroplast dry mass [51]), and thus screen the 260 nm excitation of NADPH.

3.4. Decay-associated emission spectra of reconstituted chloroplasts

Emission DAS of the BGF of reconstituted chloroplasts were also recorded both in the dark and under illumination (Fig. 5). The dark and light total fluorescence spectra were clearly different because the light-induced increase in BGF is larger in the blue than in the green. This is the expected consequence of the position of the blue fluorescence emission maximum of NAD(P)H, 460 nm for free NAD(P)H and around 440 nm for NAD(P)H bound to proteins [19,24,28,54]. But, both in the dark and under light there was an important peak around 520 nm. This peak was proposed to be a consequence of reabsorption of BGF by photosynthetic pigments [14], which is larger in the blue than in the green. Nevertheless, it appears that reabsorption is not the only reason for the presence of the green peak. The emission spectra of the C4 component (Fig. 5) shows a large peak around 530 nm, indicating the participation of other fluorophores in the green region. They are very likely flavins because the fluorescence characteristics of the 530 nm peak of the C4 component spectra are in complete agreement with those of flavins. Flavins have an absorption maximum around 370 nm [16,55] but still absorb well the 350 nm light [19]. The position of emission maximum (530 nm) is the one of flavins [16,55]. The lifetime of the long component (C4), 3.8 ns, is compatible with the fluorescence lifetimes of flavins. Free FMN has a fluorescence lifetime of about 4.7 ns [16,36,56,57] or little more [55,58,59], and free FAD has a fluorescence lifetime around 2.8 ns [36,56,57]. Still, it is known that lifetimes of flavins depend on the molecular environment. Thus, fluorescence lifetimes close to that of the C4 component have already been observed for flavoproteins [60] and free FAD [36]. In addition, the FAD bound to FNR has a lifetime of 3.9 ns for 88% of its fluorescence (Table 2). We also performed a time-resolved analysis in the dark and under light
at an excitation/emission wavelength combination (450/550 nm) that favors flavin fluorescence (Fig. 6). Flavins are known to be fluorescent in their oxidized state and non-fluorescent in their reduced state [19,61], so under reducing conditions encountered in a chloroplasts under light, a decrease of flavin fluorescence is expected. The long component (C2) is clearly decreased under light, as opposed to the shorter component which is not significantly affected (Fig. 6). We can conclude that flavin fluorescence is present in reconstituted chloroplasts, and that the main component (if not the only one) of this fluorescence has a fluorescence lifetime around 3.9 ns.

The problem of the large influence of reabsorption on the emission spectra, due to the presence of photosynthetic pigments in the chloroplast, is not alleviated in the difference spectra. Thus, as expected, in Fig. 7 we obtained a total fluorescence spectrum which is very close, in its overall shape, to the emission spectrum of NADPH. But, there are clear differences, especially around 490 nm, due to the reabsorption of BGF by photosynthetic pigments. The spectra of the lifetime components are also distorted by the reabsorption, but the spectra of the first four components still look like the spectrum of NADPH in spite of or perhaps due to the presence of a rather important noise. In addition, the maximum of the spectrum seems to be shifted towards shorter wavelengths for the components C3 and C4 compared with the components C1 and C2. This is in agreement with the above results showing that free NADPH contributes to component C1 and C2, and bound NADPH contributes to component C3 and C4, and knowing that the binding of NADPH to proteins shifts the emission spectrum of NAD(P)H about 20 nm towards shorter wavelengths [24,53,54].

3.5. Time-resolved blue-green fluorescence of intact isolated chloroplasts and comparison with reconstituted chloroplasts

The experience and results gained on reconstituted
chloroplasts permitted us to analyze the small light-induced changes in intact chloroplasts. This would have not been possible without the knowledge, obtained with reconstituted chloroplasts, that the lifetime of the components did not change significantly from dark to light, thus a global analysis was possible. Five lifetime components were also used in the time-resolved analysis of the BGF of intact chloroplasts. In order to record fluorescence decays on intact isolated chloroplasts under light, it was necessary to maintain them under conditions allowing only a very small rate of carbon metabolism. Thus, during the time of measurements, at least 10 min for only one decay, they did not change significantly. When used in the appropriate and usual conditions, intact isolated chloroplasts in solution can show rates of carbon metabolism comparable to the ones observed in vivo (e.g., [31,62,63]), but only transiently. Chloroplasts in solution depend on a restricted phosphate supply, therefore the conditions for photosynthesis are constantly changing.

Seen on Fig. 8, is the distribution of the blue fluorescence of intact chloroplasts among the different lifetime components in the dark and under light. The lifetimes of the different components are almost the same for intact like for reconstituted chloroplasts. Moreover, the excitation DAS (Figs. 3 and 9) are very similar for both types of chloroplasts. This confirms that the fluorophores are the same in intact and reconstituted chloroplasts. The only significant difference between Fig. 9 and Fig. 3 is the ratio between the 290 nm and the 340 nm peak. The 340 nm peak is proportionally larger in reconstituted chloroplasts, in which the relative content of NADPH is larger compared to proteins and to Chl. With the present very low intensity measuring beam (non-actinic), it was not possible to record a complete excitation DAS in the dark and emission DAS, either in the dark or under light, of intact isolated chloroplasts. For a large number of wavelengths the fluorescence signal is too small compared...
to the noise of the photomultiplier, and the time
needed to record these DAS would have been far
too long for the intact isolated chloroplasts.
As it was the case with reconstituted chloroplasts,
the time-resolved analysis of the BGF of intact
chloroplasts shows that the four shorter components
are all involved in the light-induced variation, where-
as the very long component (C5) is not (Fig. 8).
Furthermore, as can be seen in Table 3, there is no
significant photo-induced variations of the fractional
intensities of the first four components for intact
chloroplasts. This confirms that there is no signi-
cificant light-induced variation of the proportion of
free and bound NADPH in chloroplasts, and there-
fore that the light-induced changes of BGF in
chloroplasts is totally due to the photosynthetic re-
duction of NADP.

The distribution of fluorescence between the five
lifetime components is clearly different for reconsti-
tuted chloroplasts and intact chloroplasts (compare
Figs. 2 and 8). It is probably the result of the dilution
of the stroma in reconstituted chloroplasts, and the
resulting change in the proportion of bound NADPH in chloroplasts, and therefore that the light-induced changes of BGF in chloroplasts is totally due to the photosynthetic re-
duction of NADP.

The different parameters required for this model,
i.e., concentrations of the different compounds in in-
tact chloroplasts, and dissociation constants of the
different complexes formed, were taken from the lit-
erature (Table 4). The concentrations of all these
compounds in reconstituted chloroplasts were esti-
...
pounds: the substrate RuBP, the nocturnal inhibitor 2-carboxy-D-arabinitol 1-phosphate (CA1P) (see [65] and references therein), and other phosphoesters [50]. In the model it was necessary to take into account these ligands of Rubisco, but, as it would be too complicated to treat them all separately, we instead grouped them with RuBP, fixed at an increased concentration of 2 mM [62,63]. The same concentration of RuBP was used in the dark and under light, because the very low rate of carbon metabolism present in the chloroplasts during the time-resolved fluorescence measurements, precludes an increase in RuBP upon dark to light transitions. The dissociation constant of the complex of NADPH with Rubisco was also used for NADP⁺, because no value was found in the literature for the latter, and because the binding of NADPH with Rubisco was considered non-specific. To simplify the calculations, all other proteins that bind NADPH, apart from FNR and Rubisco which are by far the most important, were pooled together. Of course, all these proteins do not have the same dissociation constant for their complex with NADP⁺ and NADPH, so we had to use an average value for these dissociation constants. This is a reasonable simplification because the influence of this pool of ‘other’ proteins is always weak.

The entry parameters for the pool of ‘other’ proteins were obtained using the three main proteins of this pool: GAPDH, MDHAR and GR. The NADP-malate dehydrogenase (MDH) was treated alone because it is inactive in the dark and active under light [72], and these two forms have a different affinity for NADPH and NADP⁺ [70]. For all these parameters, computations were made with different values, but this did not change the main conclusions obtained in Table 5.

The results of the model (Table 5) are in agreement with the results obtained from the time-resolved measurements.

### Table 4

<table>
<thead>
<tr>
<th>Compound</th>
<th>Concentration in chloroplasts (mM)</th>
<th>(K_d) of the complex with NADPH (µM)</th>
<th>(K_d) of the complex with NADP⁺ (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NADPH</td>
<td>dark 0.5 [67]</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>light 0.7 [67]</td>
<td></td>
<td></td>
</tr>
<tr>
<td>NADP⁺</td>
<td>dark 0.4 [67]</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>light 0.2 [67]</td>
<td></td>
<td></td>
</tr>
<tr>
<td>RuBP</td>
<td>2</td>
<td>0.25b [67]</td>
<td>0.79 [68] 14 [68]</td>
</tr>
<tr>
<td>FNR</td>
<td>4e [23,64, 69]</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rubisco</td>
<td>dark 0.03e [22]</td>
<td>250 [70] 3 [70]</td>
<td></td>
</tr>
<tr>
<td></td>
<td>light 0.03e [22]</td>
<td>40d [70] 40 [70]</td>
<td></td>
</tr>
<tr>
<td>Other</td>
<td>proteinsa</td>
<td>0.065e [22]</td>
<td>85f [74] 25g [74]</td>
</tr>
</tbody>
</table>

Concentrations in the chloroplast of relevant compounds and dissociation constants of complexes were derived from the literature. For the complex of RuBP with Rubisco, a \(K_d\) of 20 µM was used [50,64,65]. Some of the data being reported in the literature in nmol per mg Chl, the volume of 25 µl per mg Chl for chloroplast stroma [66] was used to calculate the concentration. For other details see text.

aThe three other main proteins, i.e. NAD(P)-glyceraldehyde-3-phosphate dehydrogenase (GAPDH), glutathione reductase (GR) and monodehydroascorbate reductase (MDHAR), that can bind NADP⁺ and NADPH in chloroplasts, pooled together.

bAverage value calculated from different sources: 0.33 mM [22] and 0.2 mM (based on 3 FNR/PS I [71] and 600 mol Chl/mol PS I [51]).
cConcentration of active sites.
dAn average value from [70,72].
eSum of concentrations of protein binding sites: GAPDH 50 µM (mean from [22,64]), GR 1 µM [73] and MDHAR 14 µM [74].

### Table 5

<table>
<thead>
<tr>
<th>Form of NADPH</th>
<th>Intact chloroplasts</th>
<th>Reconstituted chloroplasts</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Dark (W)</td>
<td>Light (W)</td>
</tr>
<tr>
<td>Free</td>
<td>2.5</td>
<td>3.0</td>
</tr>
<tr>
<td>Bound (total)</td>
<td>97.5</td>
<td>97.0</td>
</tr>
<tr>
<td>to FNR</td>
<td>44.2</td>
<td>33.7</td>
</tr>
<tr>
<td>to Rubisco</td>
<td>52.1</td>
<td>60.5</td>
</tr>
<tr>
<td>to MDH</td>
<td>&lt;0.1</td>
<td>1.4</td>
</tr>
<tr>
<td>to other proteins</td>
<td>1.1</td>
<td>1.4</td>
</tr>
</tbody>
</table>

For details see text.
solved analysis, as they show only a slight light-induced variation of the proportion of free and bound NADPH in chloroplasts. Moreover, this variation being a decrease of bound NADPH under light, it further confirms that the light-induced changes of BGF in chloroplasts are entirely due to the photosynthetic reduction of NADP. Further, these calculations clearly showed that the main part of NADPH is bound in intact chloroplasts, which is not the case in reconstituted chloroplasts. This explains the differences in the distribution of fluorescence among the five lifetime components for reconstituted chloroplasts and intact chloroplasts, and especially, the much larger relative contribution of the C4 component and the smaller relative contribution of the C1 and C2 component to the light-induced increase in BGF in intact chloroplasts. Yet, the relative contribution of the C3 component to the light-induced increase in BGF is smaller in intact chloroplasts. This may be due to the large difference in relative distribution of bound NADPH among different proteins, especially FNR and Rubisco. This hypothesis implies that NADPH bound to Rubisco or to FNR, has different fluorescence lifetime. This assumption is strengthened by the NADPH specific binding to FNR and non-specific binding to Rubisco. Unfortunately, we could not measure the corresponding lifetimes to confirm this.

The very long component (C5) remains at almost the same level in intact and reconstituted chloroplasts (Figs. 2 and 8), so its relative contribution to total BGF is increased in intact chloroplasts (Tables 1 and 3). Thus, the fluorophore (or fluorophores) responsible for this invariable fluorescence is probably located in the thylakoid. The latter emits a low BGF when excited around 340 nm and its concentration is almost the same in our intact and reconstituted chloroplast samples.

3.6. Blue-green fluorescence of chloroplasts as a measure of NADPH concentration

To verify the linear relationship between the concentration of NADPH and the BGF in chloroplasts we had to control the NADPH concentration without varying the concentration of photosynthetic pigments (especially chlorophylls) that reabsorb this fluorescence. For this reason, it was not possible to use intact chloroplasts. To approach the optical characteristics of intact chloroplasts as accurately as possible, we used washed thylakoids fragments in the basic medium and increasing quantities of NADPH. To avoid the slow but omnipresent oxidation of NADPH by the thylakoids fragments (diaphorase activity), we inactivated the thylakoids by heating. In such a system the question remains whether the total concentration of NADPH in the measuring cell, or the NADPH concentration inside the chloroplasts, should be considered as relevant for the relation between BGF and NADPH concentration. A concentration of about 1 mM NADPH inside the chloroplasts becomes 1.25 \( \mu \text{M} \) if the total volume of a measuring cell, containing 50 \( \mu \text{g} \) Chl/ml of chloroplasts, is considered. The problem is that in front-face fluorimetry a negligible self-reabsorption is required for a linear relation between fluorescence and fluorophore concentration [20], and for NADPH in aqueous solution, this is the case only for concentration below 10 \( \mu \text{M} \) [21]. Yet, the presence in the solution of another absorbing compound, either a compound that absorbs at the excitation wavelength or at the emission wavelength or at both wavelengths, such as Chl, decreases the apparent fluorescence yield, but also increases the concentration range for the linear relationship between NADPH concentration and fluorescence [5]. More precisely, the larger the content of the absorbing compound, the larger the range of linear relationship. Anyhow, one cannot make fluorescence measurements on thylakoid fragments with NADPH in the range of NADPH concentration present inside intact chloroplasts, as it would imply using suspension of thylakoid fragments of around 22 mg Chl/ml (based on a chloroplast volume of 45 \( \mu \text{l} \) per mg Chl [66]). Therefore, we have verified the linearity between NADPH concentration and BGF in the presence of thylakoids fragments, in the concentration range corresponding to the total NADPH concentration in the measuring cell for intact chloroplasts and reconstituted chloroplasts (Fig. 10). In addition, to verify that the measurements of Fig. 10 account fittingly for the situation in intact chloroplasts, the BGF level and the light-induced variations of BGF were measured both in intact chloroplasts and in the same solution of chloroplasts after breaking of the chloroplast envelope (measurements performed on the pulse-modu-
lated fluorimeter; data not shown). The rupture of the chloroplast envelope was obtained either by osmotic shock or by freezing at -20°C. The BGF levels were almost the same, and, above all, the light-induced variations of BGF were identical. Thus, both in vitro, in concentrated solutions of chloroplasts, and by extension in vivo, there is a linear relation between the NADPH concentration and the BGF of chloroplasts.

4. Discussion

4.1. Blue-green fluorescence as a monitor of NADPH redox state in chloroplasts

Thanks to the spectral and time-resolved analysis of chloroplast BGF we obtained a strong evidence for the participation of flavins to the green fluorescence of reconstituted chloroplasts, but still no information on the nature of the flavin(s) or flavoprotein(s) responsible for this fluorescence. The FAD of FNR could be a candidate, because its main fluorescence lifetime (Table 2) is close to the one found for flavins in chloroplasts, and because FNR concentration is high in chloroplasts (Table 4). However, we found that the yield of FAD fluorescence in FNR is low (data not shown), like in other flavoproteins [16,19] and, as FNR remains bound to thylakoids, its concentration is rather low in reconstituted chloroplasts. The participation of flavins to the emitted fluorescence in the green spectral range, clearly seen here for reconstituted chloroplasts, was not detected in a previous work on intact chloroplasts [14]. This may be due to a problem of sensitivity of the time-resolved measurements of BGF, which is markedly lower for intact chloroplasts than for reconstituted chloroplasts. Another possible reason is that reconstituted chloroplasts may contain proportionally more free flavins, because of the way they are made. Further researches on this problem appear necessary to identify the flavin(s) responsible for this green fluorescence in chloroplasts. Yet, apart from this difference in flavin contribution and in the distribution of fluorescence among lifetime components, the light-induced variations of BGF were very similar for intact and reconstituted chloroplasts. Therefore, all the results obtained with reconstituted chloroplasts can reasonably be extended to intact chloroplasts.

In spite of the effect of reabsorption by photosynthetic pigments, the best emission wavelength for measuring NADPH fluorescence in chloroplasts (and by extension in algae) is around 460 nm, or may be at a little shorter wavelength, because a larger portion of NADPH is bound to proteins in intact chloroplasts than in reconstituted chloroplasts. In the blue, the light-induced variation of fluorescence intensity is at least as large as it is in the green portion of the spectrum (see [14] and Fig. 7), and it avoids the contribution from flavin fluorescence. But, for leaves, as concluded in [14] it is certainly better to use an emission wavelength in the green part of the spectrum, around 500 nm, because the potential contribution from flavin fluorescence is low, whereas the large blue fluorescence of the epidermis can be avoided.

The in vivo situation is also confronted with another problem. In leaves and in suspensions of intact...
isolated chloroplasts normally functioning (i.e., showing high rate of carbon metabolism) the concentration of RuBP highly increases under illumination (around 0.2 mM in the dark [80,81] and up to 6–7 mM under light [64,80,82]), and it may lead to a release of NADPH bound to Rubisco and then to an increase in free NADPH under light. Some of the compounds that bind to Rubisco active sites are also increased under illumination. In this case, because the fluorescence yield of free NADPH is lower than that of bound NADPH, the relation between the NADPH concentration and the BGF during dark to light transition will probably not be linear. But, some other ligands of Rubisco are decreased under illumination, in particular CA1P (K_d = 32 nM [83]), which is present at a high concentration (even higher than the Rubisco active site concentration) in some plant species in the dark and is drastically decreased upon illumination [84]. Consequently, this may compensate for the increase of RuBP, and in this case the quantity of NADPH bound to Rubisco would not significantly change between dark and light, but it would always remain very low. There is still another aspect of this problem to be considered. Binding of NADPH to Rubisco is non-specific, and as the fluorescence of NADPH is not always enhanced when bound to proteins [19] or when bound to a non-specific site [85], it is still possible that this binding does not significantly change the NADPH fluorescence yield. Unfortunately, our attempts to measure the fluorescence yield and lifetime of NADPH when bound to purified Rubisco were unsuccessful, because of the presence of inherent UV-A induced blue fluorescence in two different types of purified Rubisco. Thus, the influence of the RuBP changes on the linear relation between light-induced changes of BGF and the corresponding changes of NADPH concentration in leaves and optimally functioning chloroplast suspensions, remains open. In this situation, the concentration of NADPH bound to Rubisco can only be lower, and not higher, under illumination. Therefore the light-induced increase in chloroplast BGF can only come from an increase in NADP* reduction. The new data presented in this work confirm this relationship in isolated chloroplasts and, by extension, in chloroplasts in vivo. The blue-green fluorescence can therefore be used confidently for non-destructive, continuous and probably quantitative monitoring of the light-induced changes of NADP redox state.

4.2. Energy transfer from proteins to NADPH in chloroplasts

The combination of NADPH specific light-induced variation of chloroplast BGF with the time-resolved and spectral analysis of this fluorescence proved to be a very powerful approach. It permitted not only to demonstrate that there is no significant light-induced variation of the proportion of free and bound NADPH in chloroplasts, but also revealed the existence of an energy transfer between proteins and NADPH. This energy transfer is necessary to explain the 286 nm peak in the difference excitation fluorescence spectrum of reconstituted chloroplasts (Fig. 4), because, as it is a difference spectrum between light and dark, NADPH is the only fluorophore involved in this spectrum but it has no absorption (excitation) peak at 286 nm. Moreover, this peak cannot be the 260 nm excitation peak of free NADPH being displaced towards longer wavelengths for a yet unknown reason, because it is not present in the difference spectrum of the C1 and C2 lifetime components. The peak at 286 nm corresponds to the absorption of proteins, more precisely, to the maximum of absorption of tryptophan (Trp) [16]. So, the energy transfer is most probably between Trp residues and NADPH. This energy transfer can be of a non-radiative type, Förster resonance energy transfer, or of a radiative type, with NADPH reabsorbing the fluorescence emitted by proteins at 350 nm, or a mix of the two types. Resonance energy transfer notably implies a very short distance between the donor and the acceptor. Therefore, this type of transfer can occur only to bound NAD(P)H. This transfer is known to occur in proteins between Trp and bound NAD(P)H [19]. It was reported in several proteins [27,48,49,86], and was even used for distance determination in other proteins [54]. The putative radiative transfer to NADPH involves also mainly Trp, because the fluorescence of the other amino acids is negligible compared with Trp fluorescence [16]. Fortunately, by using the fluorescence lifetime we can differentiate between these two types of transfer. The non-radiative transfer is a de-excitation process of the excited
state of Trp residues in competition with all other de-excitation processes, especially fluorescence. In order to be efficient this transfer has to be faster than fluorescence (mean lifetime around 2.8 ns, Table 2). In the case of radiative transfer, an emission of fluorescence by the donor is followed by re-absorption and re-emission of fluorescence by the acceptor, therefore the fluorescence lifetime is the sum of the fluorescence lifetime of the donor (Trp) and the acceptor (NADPH). The lifetime of the C3 component (2.1 ns) is too short to explain the presence of its 286 nm peak by radiative transfer. So part of the energy transfer between the proteins and NADPH must be of the non-radiative type. Thanks to the presence of the 340 nm peak in the difference spectrum, we know that the C4 component (5.6 ns) contains NADPH bound to proteins with a long fluorescence lifetime. Thus, a non-radiative energy transfer most probably contributes to the 286 nm peak of the C4 component. Yet, the ratio of the 286 and 340 nm peak are clearly different for the C3 and C4 component, which seems incompatible with non-radiative energy transfer only. Indeed, the population of bound NADPH molecules excited directly (340 nm peak) or excited by resonance energy transfer (286 nm peak) should be the same, because this transfer is fast and the fluorescence lifetime, even after transfer, is mainly that of bound NADPH. In the case of the C3 component, in which only non-radiative energy transfer occurs, the fluorescence yield in the difference spectrum, that include non-radiative transfer (286 nm peak), is almost equal to the fluorescence yield of direct excitation (340 nm peak). Thus, if only non-radiative transfer is involved, the corresponding 286 nm peak of the C4 component should have almost the same height as the 340 nm peak. By contrast, the 286 nm peak of the C4 component is much higher, implying the contribution of radiative transfer which is possible here because the lifetime of the C4 component is sufficiently long to account for it. For the radiative transfer the acceptor can be free NADPH as well as bound NADPH. After transfer, the fluorescence of free NADPH appears in the C4 component because of proteins fluorescence lifetime, but of course, it appears in the C1 and C2 components under direct excitation. In addition, the presence of the resonance energy transfer, between proteins and bound NADPH in the C3 and C4 lifetime components, is a confirmation that fluorescence of bound NADPH participates to the C3 and C4 components.

Acknowledgements

We would like to acknowledge the support of the Centre National de la Recherche Scientifique (France) through the GDR 1536 ‘FLUOVEG’. The authors wish to thank Dr M. Miginiac-Maslow for the gift of FNR, Dr M. Parry for the gift of Rubisco, Dr F. Merola for help in analyses by the maximum entropy method, and Dr Y. Goulas for valuable discussions and the programming of the model. Thanks are also due to Dr O. Bonham-Carter and Dr S. Meyer for checking the English text.

References