LOW-TEMPERATURE (4-77°K) SPECTROSCOPY OF ANACYSTIS; TEMPERATURE DEPENDENCE OF ENERGY TRANSFER EFFICIENCY

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SUMMARY

From the absorption spectra, emission and excitation spectra of chlorophyll a (Chl a) and phycocyanin fluorescence measured (from 4 to 77°K) at different wavelengths, we confirmed that a large portion of Chl a (Chl a 670, Chl a 678 and Chl a 705), in Anacystis, is in System I, and that System II is composed mainly of phycocyanin and of some Chl a 670 and Chl a 678. Our data also confirmed the following correspondence between absorption and fluorescence bands: phycocyanin (with bands at 580, 625 and 634-637 nm) and allophycocyanin (approx. 650 nm) are responsible for the broad fluorescence band at 653-655 nm and Chl a 670 for F680 (when Chl a 678 is preferentially extracted). (Date in ref. 20 show that Chl a 678 is responsible for F685.) The ratio of f715 to f685 + f695 was greater in System I than in System II.

The efficiency of energy transfer from Chl a 670 to Chl a 678, that approaches 100 % even at 4°K, was found to be independent of temperature. However, transfer efficiency from phycocyanin to Chl a was temperature dependent; it was lower at 4 than at 77°K. The quantum yield of Chl a fluorescence (λ excitation = 435 nm) was higher (by a factor of 4) at 4 than at 77°K. The estimated quantum yield of fluorescence in Anacystis at 4°K is only 6 % (λ excitation = 560 nm) and approx. 3 % (λ excitation = 436 nm).

INTRODUCTION

Blue-green algae are of interest in photosynthetic research because of their uniqueness: (1) they are prokaryotic; (2) they do not have much of a dark respiration; (3) the two pigment systems are spectrally well separated and (4) growth conditions affect dramatically their pigment systems and the efficiencies of energy

Abbreviations: The prefix f will be used for the fluorescence intensity at a certain wavelength, and F when referring to fluorescence bands F650, F680, F685, F695 and F710 even though their exact locations are a few nm off. Chl, chlorophyll.

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transfer\textsuperscript{12–14}. Measurements\textsuperscript{15–19} of fluorescence spectra of blue-green algae at $77^\circ$K have revealed the presence of three chlorophyll $a$ (Chl $a$) emission bands at 685 nm (F685), 695 nm (F695) and 712 nm (F710). Excitation spectra of fluorescence at $77^\circ$K for f690, f700 and f720 in the 400–650-nm range are known for whole cells\textsuperscript{17,19}, and for f720 in the 550–690-nm range for Anacystis fragments prepared by digitonin treatment and ultracentrifugation\textsuperscript{18}. (For references to earlier literature on fluorescence, see refs. 20 and 21.)

Using absorption spectra at 4 and $77^\circ$K (that are different from those at room temperature) and the excitation spectra for f653, f687, f698 and f715 measured at different temperatures ($4$–$77^\circ$K), we have calculated the efficiencies of energy transfer as a function of temperature. Our data suggest that the energy transfer from phycocyanin to Chl $a$ is temperature dependent. However, the efficiency of energy transfer from Chl $a$ 670 to Chl $a$ 678 is independent of temperature.

**MATERIALS AND METHODS**

*Anacystis nidulans* was grown as described earlier\textsuperscript{5}. The absorption spectra and the excitation spectra of fluorescence at low temperatures ($4$–$77^\circ$K) were measured as described in the previous paper\textsuperscript{20}. In the absorption spectra measurements, the fluorescence caused by the incident light was removed by passing the collected (fluorescence and transmitted) light through a second monochromator; however, in the region of the overlap of the emission and the absorption spectra, fluorescence cannot be separated from the transmitted light.

Fluorescence spectra were measured as described earlier\textsuperscript{22}. For these spectra, all slits had half band widths of 6.6 nm, and a colored glass filter C.S. 4-94 (Corning, N.Y.) was placed between the exciting monochromator and the sample, whereas C.S. 2-73 was placed between the sample and the analyzing monochromator. All the fluorescence spectra were corrected for the transmission efficiency of the monochromator (and filters) and the spectral distribution of the photomultiplier. For further details, see ref. 23.

**RESULTS**

*Absorption spectra of A. nidulans at 4 and $77^\circ$K*

Low-temperature absorption spectra for blue-green algae are not found in the literature. In Anacystis, the 4 and $77^\circ$K absorption spectra are very similar (deviation is less than 5\%) except that the bands at $4^\circ$K show slightly better resolution (Fig. 1). It must be emphasized, however, that there is no shift in the absorption peaks when $4^\circ$K spectra are compared with the $77^\circ$K spectra. This information is important for our conclusions on the transfer efficiencies.

In comparison with the room temperature spectrum, the $4^\circ$K spectrum shows the following differences (Table I; Fig. 1): (a) The spectrum of carotenoids is distinctly resolved into two bands at 465 and 502 nm (cf. ref. 35). (b) The phycocyanin band is resolved into two peaks at 625 nm and 634 nm and a shoulder in the 580–600-nm range is present; a band at 650 nm caused by allophycocyanin is also observed. (c) The Chl $a$ band is resolved into two peaks at 670 nm (Chl $a$ 670) and at 679 nm (Chl $a$ 679), and two shoulders at 686 nm (Chl $a$ 686) and 705–710 nm (Chl $a$ 705).
Fig. 1. Absorption spectra of *A. nidulans* measured at 4°K (○), 77°K (△), and 295°K (●).

**TABLE I**

**THE ABSORPTION PEAKS OF ANACYSTIS AT DIFFERENT TEMPERATURES**

S = shoulder, P = peak. Values in nm.

<table>
<thead>
<tr>
<th></th>
<th>295°K</th>
<th>77°K</th>
<th>4°K</th>
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<tbody>
<tr>
<td><strong>In vivo</strong></td>
<td>295°K</td>
<td>77°K</td>
<td>4°K</td>
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<tr>
<td>Chl a</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Red region</td>
<td>679 (P; broad)</td>
<td>670 (S)</td>
<td>670 (S) (slightly sharper than at 77°K)</td>
</tr>
<tr>
<td></td>
<td>679 (P)</td>
<td>679 (P)</td>
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<tr>
<td></td>
<td>686 (S)</td>
<td>686 (S)</td>
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<tr>
<td></td>
<td>705 (S)</td>
<td>705 (S)</td>
<td></td>
</tr>
<tr>
<td>Blue region</td>
<td>750 (S)</td>
<td>745-750 (S)</td>
<td>745-750 (S)</td>
</tr>
<tr>
<td>Phycocyanins</td>
<td>440 (P)</td>
<td>440 (P)</td>
<td></td>
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<tr>
<td></td>
<td>630 (S; due to allophycocyanin)</td>
<td>630 (S; due to allophycocyanin)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>635 (S; not very clear)</td>
<td>634 (P)</td>
<td>634 (P) (slightly sharper than at 77°K)</td>
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<tr>
<td></td>
<td>625 (P)</td>
<td>625 (P)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>580 (S; not very clear)</td>
<td>580 (P or S)</td>
<td>580 (P or S)</td>
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<tr>
<td>Carotenoids</td>
<td>490-505 (S)</td>
<td>502 (P) (cf. ref. 35)</td>
<td>502 (P)</td>
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<tr>
<td></td>
<td>460-465 (S)</td>
<td>465-470 (S)</td>
<td>465 (P)</td>
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</table>

are not easily recognized without analysis of the fluorescence excitation spectra; a third clear shoulder at 750 nm (P750N), noted before at room temperature, is also seen.

**Fluorescence spectra**

The fluorescence spectra of Anacystis are very different depending upon the excitation wavelength. At room temperature, excitation at 560 nm causes...
a broad phycocyanin fluorescence band at 653 nm and a shoulder near 680–685 nm (Chl a). Excitation at 435 nm produces a very weak fluorescence band near 685 nm. When the cells are cooled to 77 °K, the fluorescence intensity increases, and additional bands at 696 nm and 715 nm appear. (The 696-nm band was first noted by Litvin et al. in leaves, and the 715-nm band was discovered by Brody in Chlorella.)

Fig. 2 shows the fluorescence spectra of Anacystis excited at 560 nm as a function of temperature (4–77 °K); the room temperature spectrum is given for comparison. At 4 °K, the broad F710 band has a peak at 712 nm. The total fluorescence intensity

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increases 4–5 times upon cooling from 295 to 4°C. However, since the absorption increases by approx. 100% at 560 nm (see Fig. 1), there is only a 2-fold net increase of the fluorescence yield. Thus for λ excitation = 560 nm, the yield at 4°C is approx. 6%, since the yield at 20°C is approx. 3%.

Fig. 3 shows the low-temperature (4–77°C) fluorescence spectra of Anacystis when excited at 435 nm. Under this condition, the fluorescence intensity at 712–715 nm increases 15–17-fold upon cooling to 77 and 70-fold when cooled to 4°C. However, increases in f685 and f695 are much lower, and there is a 30% increase in the absorption at 435 nm. For λ excitation = 435 nm, the net increase in the total yield (including all the three fluorescence bands) is 13–14 times that at room temperature; the yield at 4°C is approx. 3%, because the yield at room temperature is only 0.2% (ref. 27).

Fig. 4 shows the excitation spectra for f653 at six different temperatures (4, 24, 43, 62, 71, and 79°C). In the 4°C spectrum, bands at 580, 623 and 637 nm due

Fig. 4. Excitation spectra for f653 in A. nidulans in the 4–79°C range.

Fig. 5. Excitation spectra of phycoyanin fluorescence in vitro at 77 and 295°C.

to phycocyanin are observed. This spectrum suggests that Chl a is not responsible for F65o because no band at 435 nm was observed. The relative heights among these bands remain almost constant upon warming from 4 to 77 °K. Fig. 5 shows excitation spectra of pure phycocyanin at 77 and 295 °K for comparison.

Fig. 6 shows the excitation spectra for f687 at six different temperatures (4, 21, 44, 60, 71 and 77 °K). Bands at 580, 623 and 637 nm due to phycocyanin are again observed; a shoulder near 650 nm due to allophycocyanin is seen. In the blue region, a small Chl a band (near 436 nm) is also observed. The ratio of 637 nm (phycocyanin) to 436 nm (Chl a) band is about 9 at 4 °K. This shows that System II is composed mainly of phycocyanin and a very small amount of Chl a, since we assume that most of F685 is from System II (ref. 21).

Fig. 6. Excitation spectra for f687 in A. nidulans in the 4-77 °K range.

Fig. 7 shows the excitation spectra for f715 at five different temperatures (4, 31, 53, 68 and 79 °K). The Chl a excitation bands at 440, 670 and 678 nm and a shoulder at 686 nm (not clearly resolved) are seen. (The positions of the bands for carotenoids and phycocyanin are similar to those found in the excitation spectra for f698 — not shown here.) At 4 °K, the ratio of the excitation band at 637 nm (phycocyanin) to that at 436 nm is lower in the excitation spectrum for f715 than that for f698 and f687. Higher Chl a excitation bands for f715 than for f687 and f698 have previously been discussed suggesting that the long wave fluorescence belongs preferentially to System I. We know that the emission spectrum excited by 560 nm belongs preferentially to System II because 560-nm light is preferentially absorbed by System II. The relative heights of the excitation bands are highly temperature dependent; analyses will be shown later. The spectra obtained in the present study are slightly better resolved than those reported at 77 °K (refs. 16 and 17); the 637-nm band found in this study (in some cultures, this band was found at 633 nm) has not been

LOW TEMPERATURE SPECTROSCOPY OF ANACYSTIS

reported before\textsuperscript{16,17}, although the general shapes of the excitation spectra of these studies are similar.

The relationship between Chl \(a\) molecules and the fluorescence at 687, 697 and 712 nm was studied with Anacystis which had been repeatedly extracted with 80\% acetone. The absorption spectrum of this sample showed that only about 5-10\% of the Chl \(a\) molecules remained in the cells and the red absorption band had mainly the shorter wavelength form of Chl \(a\) (Chl \(a\) 670). Fig. 8 shows the 77\°K fluorescence spectra of these cells excited at 435 and 560 nm. The most obvious difference between these spectra and those of the normal cells (Figs. 2 and 3) is that the long wavelength

\hspace{1cm}

![Fig. 7. Excitation spectra for f715 in \textit{A. nidulans} in the 4–79\°K range: \(\triangle\), 4\°K; \(\bullet\), 31\°K; \(\square\) 53\°K; \(\blacktriangle\), 68\°K; \(\bigcirc\), 79\°K; values beyond 690 nm are partly caused by the leakage of light.]

![Fig. 8. Fluorescence spectra of resuspended \textit{A. nidulans} from which 90–95\% chlorophyll \(a\) (primarily the longwave forms of Chl \(a\) including Chl \(a\) 680) has been extracted: temperature, 77\°K; \(\bullet\), \(\lambda\) excitation 435 nm; \(\blacktriangle\), \(\lambda\) excitation 560 nm.]

(>700 nm) fluorescence is greatly reduced in the Chl a-extracted cells, confirming that the long wavelength fluorescence bands are caused by longwave forms of Chl a molecules. Furthermore, when the fluorescence spectrum excited at 435 nm is compared with that excited at 560 nm, the former shows a higher f681–760 to f653 ratio; this indicates that the fluorescence from 681 to 760 nm is caused by the Chl a molecules.

We also note that in the 80 % extracted samples an emission peak at 655 nm is present in the 435-nm-excited spectrum, whereas in normal samples (Fig. 3) there is no such peak. This is because phycocyanin does have some absorption at 435 nm, and it is relatively more abundant than Chl a in the treated samples. We cannot, however, rule out the possibility that there may be some “uphill” energy transfer (cf. ref. 34) from Chl a to phycocyanin. But, we consider it unlikely.

**DISCUSSION**

The molecular origin of fluorescence bands in Anacystis

Figs. 2 and 3 show the fluorescence bands at 653 (f65o), 681 (f68o), 685–687 (f685), 696 (f695) and 712 (f710) nm. Additional shoulders at 725, 740 and 755 nm are not clearly resolved. The positions of these bands are similar to those found in Chlorella: the phycocyanin fluorescence band at 653 nm is, however, not found in Chlorella simply because the latter does not contain phycocyanin.

The f65o is a phycocyanin fluorescence band; this is indicated by the similarity between the absorption spectrum of phycocyanin \textit{in vivo} (Fig. 1) and the excitation spectrum of F65o \textit{in vivo} (Fig. 4) and \textit{in vitro} (Fig. 5). The excitation spectrum of the extracted phycocyanin shows excitation bands at 580, 625 and 660 nm (at 77°K) as compared to the 580, 623 and 637 nm found in Anacystis.

The fluorescence band at 681 nm (f68o) found in 80 % acetone-extracted Anacystis (Fig. 8) is suggested to be due to Chl a 670 that are close to the phycocyanin molecules. The energy cannot be transferred any further to Chl a 678 after 90 % of the Chl a has been extracted, and the fluorescence from Chl a 670 occurs. These findings and those of CEDERSTRAND \textit{et al.} confirm the suggestion that Chl a 670 emits near 680 nm, when Chl a 678 is preferentially extracted. This conclusion fits very well with the finding that Chl a 678 emits near 687 nm. The Chl a 695–705 emits near 712 nm (ref. 30).

Irreversible energy transfer from phycocyanin to chlorophyll a — resonance transfer

In Anacystis, excitation of phycocyanin causes fluorescence not only from phycocyanin but also from Chl a (Fig. 2) showing energy transfer from phycocyanin to Chl a even at 4°K. (Similar conclusions at room temperature and 77°K were made by other authors.

When Chl a or carotenoids are excited, only Chl a but no phycocyanin fluorescence is found, suggesting that energy can only be transferred from phycocyanin to Chl a but not in the reverse direction. This irreversible energy transfer pathway satisfies the condition for the resonance transfer model which was discussed in detail by FORSTER and by ROBINSON. Furthermore, according to this model, the transfer rate may vary as a function of temperature, because the amount of band overlap may change upon cooling as a result of sharpening and shifting of the absorption and

OR the emission bands. It will be shown below that the energy transfer from phyco-
cyanin to Chl a indeed obeys this condition.

The energy transfer efficiencies and their temperature dependence in Anacystis
were analyzed by the same methods as those in Chlorella (see APPENDIX in ref. 20).

There can be a general criticism of comparing transfer efficiencies at different
temperatures if absorption data are not considered with caution. One has to be quite
sure whether even minor shifts in the absorption peaks occur. As the slopes of these
spectra are rather steep at low temperature, a small shift could cause a considerable
change in the number of absorbed quanta and, just for this reason fluorescence
intensity may change, whereas the fluorescence yield may be unchanged. Such shifts
do not occur from 4 to 77°K (see Fig. 1). Moreover, if they did we could calculate the
true efficiencies simply because we have reliable absorption data.

The estimation of the transfer efficiency from allophycocyanin (or phycocyanin)
to Chl a is attempted in a similar way as from Chl b to Chl a (ref. 20). Unfortunately
the ratios $A_{670\,\text{nm}} / A_{650\,\text{nm}}$ for System I and System II are not certain for Anacystis;
therefore, the absolute transfer efficiencies cannot be obtained. The ratio of the total
absorbance at 650 and 670 nm is constant in the 4–77°K range. With assumptions
similar to those used for Chlorella, the temperature dependence of the transfer ef-
ficiencies was estimated as follows:

For fluorescence at 715 nm (preferentially System I),

$$\frac{\left[R_{650 \rightarrow 670}\right]_4}{\left[R_{650 \rightarrow 670}\right]_{77\,\text{K}}} = \left[\frac{E_{650}^{715}}{E_{670}^{715}}\right]_4 \left[\frac{E_{650}^{715}}{E_{670}^{715}}\right]_{77\,\text{K}} = 0.90$$  \hspace{1cm} (1)

For fluorescence at 698 nm (preferentially System II),

$$\frac{\left[R_{650 \rightarrow 670}\right]_4}{\left[R_{650 \rightarrow 670}\right]_{77\,\text{K}}} = \left[\frac{E_{650}^{698}}{E_{670}^{698}}\right]_4 \left[\frac{E_{650}^{698}}{E_{670}^{698}}\right]_{77\,\text{K}} = 0.80$$  \hspace{1cm} (2)

Eqns. 1 and 2 show the increase of transfer efficiency from phycocyanin (and allo-
phycocyanin) to Chl a (mainly in System I) upon warming from 4 to 77°K; Eqns. 3
and 4 show the increase of transfer efficiency under the same conditions for both Sys-
tems I and II. An average increase of 20% is found for the transfer efficiency from
phycocyanin to Chl a upon warming from 4 to 77°K in all cases. This result suggests
that energy transfer among these heterogeneous molecules obeys the resonance transfer
model, which is in agreement with the theoretical predictions.

Energy transfer from Chl a 670 to longwave forms of Chl a

Assuming that energy absorbed by Chl a 670 is first transferred to Chl a 678
and then to longer wavelength Chl a forms and is emitted at 715 nm, then, the equa-

tion in ref. 20, with slight modifications, will be applicable to the calculation of the transfer efficiency in Anacysitis. The modified equation is:

\[ R'_{670 \rightarrow 678} = \frac{E'_{678}}{E'_{670}} \frac{A'_{678}}{A'_{670}} \]  

(5)

where \( R'_{670 \rightarrow 678} \) is the transfer efficiency from Chl a 670 to Chl a 678 in System I. \( E'_{670} \) and \( E'_{678} \) are the System I fluorescence intensities measured at 715 nm, excited at 670 and 678 nm, respectively. \( A'_{678} \) and \( A'_{670} \) are the quanta absorbed by System I at 678 and 670 nm, respectively.

Since most of the Chl a of Anacystis is in System I (shown by the action spectrum of the two light reactions in photosynthesis\(^9\)) and since excitation at the Chl a absorption bands causes a strong fluorescence band at 712–715 nm and weak bands at 685 and 695 nm (Fig. 3), \( E'_{675}/E'_{715} \) is indicated by the ratio of the excitation band heights for F710 (Fig. 7), whereas \( A'_{678}/A'_{670} \) can be determined from the ratio of the absorption band heights of Chl a 678 and Chl a 670 shown in Fig. 1. This is allowable because the concentration of the sample for measuring the excitation spectrum was very low (absorbance 0.1–0.2 at 675 nm); therefore, the ratio of the absorbed quanta can be estimated by the ratio of the absorption band heights. Calculations show that the transfer efficiency from Chl a 670 to Chl a 678 approaches 100% (allowing 5% error), and this efficiency is independent of temperature from 4 to 77 K. This can be interpreted to mean that if the number of transfers among the homogenous molecules (Chl a 670) is large (for example, \( 10^4 \)), then the slow (1–100 psec) transfer mechanism within Chl a 670 is not possible because it would take 0.01–1 µsec before energy is dissipated, but we know that this is not the case since the measured life-time of the excited state in vivo is approx. 1 nsec. However, if the transfer number is very small, the irreversible transfer from Chl a 670 to Chl a 678 will lead to the observed result regardless of the transfer rate (fast (0.01–1 psec) or slow (1–100 psec)) among the homogeneous molecules because the transfer rate from Chl a 670 to Chl a 678 would still be faster than the dissipation of energy from Chl a 678.

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