Efficient biosynthetic incorporation of tryptophan and indole analogs in an integral membrane protein

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Abstract

Biosynthetic incorporation of tryptophan (Trp) analogs such as 7-azatryptophan, 5-hydroxytryptophan, and fluorotryptophan into a protein can facilitate its structural analysis by spectroscopic techniques such as fluorescence, phosphorescence, nuclear magnetic resonance, and Fourier transform infrared. Until now, the approach has dealt primarily with soluble proteins. In this article, we demonstrate that four different Trp analogs can be very efficiently incorporated into a membrane protein as demonstrated for the mannitol transporter of *Escherichia coli* (EII^{mtl}). EII^{mtl} overexpression was under control of the λP_R promoter, and the *E. coli* Trp auxotroph M5219 was used as host. This strain constitutively expresses the heat labile repressor protein of the λP_R promoter. Together with the presence of the repressor gene on the EII^{mtl} plasmid, this resulted in a tightly controlled promoter system, a prerequisite for high Trp analog incorporation. A new method for determining the analog incorporation efficiency is presented that is suitable for membrane proteins. The procedure involves fitting of the phosphorescence spectrum as a linear combination of the Trp and Trp analog contributions, taking into account the influence of the protein environment on the Trp analog spectrum. The data show that the analog content of EII^{mtl} samples is very high (>95%). In addition, we report here that biosynthetic incorporation of Trp analogs can also be effected with less expensive indole analogs, which in vivo are converted to L-Trp analogs.

Keywords: Tryptophan analog; biosynthetic incorporation; alloprotein; phosphorescence spectroscopy; membrane protein

Tryptophan (Trp) is an attractive intrinsic spectroscopic probe in protein chemistry (Lakowicz 1999). Its fluorescence and phosphorescence emissions are sensitive to the microenvironment and provide high-resolution, site-specific

information about protein structure and dynamics. The potential of this probe has been expanded by genetic techniques that make possible the construction of single-Trp-containing proteins and permit the introduction of the probe at the desired residue position. The use of alloproteins, proteins containing an unnatural amino acid, further expands the scope of Trp spectroscopy in protein chemistry. A number of Trp analogs can be biosynthetically incorporated, including 7-azatryptophan (7-ATrp), 5-hydroxytryptophan (5-OHTrp) and fluorotryptophans (Ross et al. 1997). The Trp analogs 7-ATrp and 5-OHTrp are of particular interest because of their red-shifted absorption spectrum. Both analogs can be excited at wavelengths up to 315 to 320 nm, 20 nm to the red of Trp absorption. Biosynthetic incorporation of these analogs in a given protein makes it possible to selectively excite the alloprotein in a high Trp background. Likewise, selective monitoring by absorption spectroscopy of the alloprotein is possible also in the presence of nucleic

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Abbreviations: EII^{mtl}, the mannitol-specific transporting and phosphorylating enzyme from *Escherichia coli*; Mtl, mannitol; Trp, tryptophan; 1-MTrp, 1-methyltryptophan; 4-FTrp, 4-fluorotryptophan; 5-FTrp, 5-fluorotryptophan; 5-OHTrp, 5-hydroxytryptophan; 7-ATrp, 7-azatryptophan; 4-Indole, 4-fluoroindole; 5-Indole, 5-fluoroindole; 5-OHindole, 5-hydroxyindole; C₁₀E₅, decylpenta (ethylene glycol); NATA, *N*-acetyl-L-tryptophanamide; PG, 1,2 propylene glycol; BW, bandwidth in nm of the 0,0 vibronic band at two-thirds peak height; $\lambda_{0,0}$, peak wavelength of the 0,0 vibrational band; λ_{ex} , excitation wavelength; ϕ_p , phosphorescence quantum yield.

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acids, absorbing strongly in the UV. Protein–DNA interactions have been studied after incorporation of 7-ATrp or 5-OHTrp in the protein (Ross et al. 1997). Fluorotryptophans with a fluoro atom at the fourth, fifth, or sixth position have been used for decades as probes in ^{19}F nuclear magnetic resonance (NMR). 4-Fluorotryptophan (4-FTrp) is nonfluorescent and can therefore be used to generate fluorescence free proteins without requirement to replace the Trp codons at the genetic level.

A number of steps and/or techniques need to be carefully tuned for a profitable application of Trp analog–containing proteins in spectroscopy. There is need for protocols that guarantee high analog incorporation efficiency into target proteins, as well as methods to quantify the degree of incorporation. Biosynthetic labeling of the target protein with Trp analogs is usually achieved with an *Escherichia coli* Trp auxotroph, transformed with a plasmid encoding the protein under control of an inducible promoter (Hogue et al. 1992; Ross et al. 1992, 1997). The auxotroph is grown in a medium containing Trp, and when enough biomass has been generated, the cells are collected by centrifugation. Protein expression is induced after resuspending the washed cells in a medium containing the Trp analog. The analog incorporation efficiencies reported for different proteins vary from <30% to >95% (Ross et al. 1997). Although the wide variation in analog incorporation efficiency is not well understood, the use of a leaky promoter, resulting in the expression of the target protein during the growth in the Trp-containing medium, is one factor leading to lower analog incorporation. The most straightforward approach to quantify the Trp analog incorporation efficiency is through the deconvolution of the absorption spectrum of the alloprotein, in terms of the parent spectra of Trp and of the Trp analog (Waxman et al. 1993).

To date, biosynthetic incorporation of Trp analogs has been concentrated on water-soluble proteins (Ross et al. 1997). The potential of this approach may be even larger for the biophysical characterization of integral membrane proteins. Under in vivo conditions, these proteins are often functional as oligomers or complexed to other (membrane) proteins, and such noncovalent interactions can be lost or weakened upon extraction with detergent and subsequent purification. To study membrane proteins in their natural environment, we have incorporated 7-ATrp into single-Trp–containing mutants of the mannitol transporter (EII^{mtl}) of *E. coli* (Broos et al. 1999). By selective red-edge excitation of the probe, as well as the characteristic change in fluorescence intensity, mannitol binding by these 7-ATrp–containing mutants could be monitored directly in *E. coli* vesicles and even in whole cells. In combination with the stopped-flow technique, the pre-steady-state binding kinetics of mannitol and binding of an inhibitor could be studied (Broos et al. 1999).

Here we report a methodology resulting in a very high to absolute Trp-analog incorporation efficiency for different analogs in EII^{mtl}. Expression of EII^{mtl} was under control of the heat-inducible λP_R promoter (van Weeghel et al. 1990). The utility of this promoter, together with the M5219 Trp auxotroph (Remaut et al. 1981), an *E. coli* host with a chromosomal insertion encoding the λ cI₈₅₇ heat labile repressor protein, is discussed. Estimation of the Trp analog incorporation efficiency via deconvolution of the absorption spectrum of the alloprotein was found not suitable for membrane proteins. A new method was adopted based on phosphorescence emission of the probe. We also explored the possibility to incorporate Trp analogs via their indole analogs. A procedure was developed which makes use of the ability of the *E. coli* host to convert indole into Trp. This procedure can be successfully applied in the case of fluorotryptophans because the incorporation efficiencies were as high as those starting from Trp analogs.

Results

Incorporation of Trp analogs into W30

Trp analog incorporation into the integral membrane protein EII^{mtl} was explored by using the single-Trp-containing mutant W30, a construct in which Trp residues at position 42, 109, and 117 have been replaced with phenylalanine (Swaving Dijkstra et al. 1996a). The Trp analogs 7-ATrp, 5-OHTrp, and 5-fluorotryptophan (5-FTrp) were biosynthetically incorporated into W30, and the proteins were purified to >95% homogeneity by using metal affinity- and ion exchange chromatography. All three alloproteins were functional because they exhibited mannitol-binding affinities and mannitol phosphorylation activities comparable to those of W30 or wild-type EII^{mtl}-containing Trp (Swaving Dijkstra et al. 1996a). Fluorescence excitation spectra of the different purified W30 proteins, presented in Figure 1, show that all three Trp analog–containing samples are red-shifted relative to the Trp-containing sample. Comparison with published absorption spectra of the analogs (Ross et al. 1997; Wong and Eftink 1998) indicate that the incorporation efficiency in each sample is very high.

The low-temperature phosphorescence emission spectra of the W30 alloprotein samples in 1,2 propanediol (PG)/buffer glasses are presented in Figure 2. The spectra of W30 with the various analogs differ markedly, and comparison with the spectra of free analogs (Fig. 3A) indicates a high degree of incorporation in each sample.

Quantitative estimation of the Trp analog incorporation efficiency by low-temperature phosphorescence

The method proposed here to determine the Trp/Trp analog composition of a protein sample is based on the differences

Figure 1. Fluorescence excitation spectra of W30 (solid line) and W30 alloproteins containing 5-FTrp (dashed line), 5-OHTrp (dotted line) and 7-ATrp (dashed and dotted line). The emission wavelength was 350 nm. Spectra were normalized to their maxima.

between the low-temperature phosphorescence spectra of Trp and that of the analogs (Fig. 3A; Wu et al. 1996; Cioni et al. 1998; Ozarowski et al. 1998). A characteristic feature of low-temperature Trp phosphorescence spectra is the well-resolved vibrational structure with an intense band at the blue side of the spectrum, representing the transition to the lowest vibrational level of the ground state (0-0 vibronic band). Its wavelength and bandwidth are a sensitive monitor of the polarity and structural homogeneity of the Trp microenvironment, respectively (Hershberger et al. 1980; Strambini 1989; Vanderkooi 1992; Schauerte et al. 1999). A preliminary test of the performance of spectral deconvolution was carried out by using known mixtures of the free chromophores in solution. At each wavelength, λ , the phosphorescence intensity of the mixture $I_p(\lambda)$ was fitted (Fig. 3B–D) by using a linear combination of the spectra of the pure chromophores obtained under the same excitation conditions (295 nm) according to Equation 1:

$$I_p(\lambda) = \alpha \times I_p^W(\lambda) + (1 - \alpha) \times I_p^{An}(\lambda) \times A_r^{An} \quad (1)$$

$I_p^W(\lambda)$ and $I_p^{An}(\lambda)$ are the emission intensities of free Trp (*N*-acetyl-L-tryptophanamide [NATA]), and of the Trp analog, respectively, normalized to unity spectral area. $A_r^{An} = \int I_p^{An}(\lambda) / \int I_p^W(\lambda)$, the ratio of the area under the spectra of equimolar analog and NATA solutions and is equal to the relative phosphorescence quantum yield, ϕ_p^{An} , multiplied by the extinction coefficients of the analog over Trp at 295 nm. For 5-FTrp $A_r = 1.68$ and $\phi_p^{An} = 0.60$, for 5-OHTrp $A_r = 1.13$ and $\phi_p^{An} = 0.39$, and for 7-ATrp $A_r = 0.14$ and $\phi_p^{An} = 0.036$. α represents the fraction of Trp in the mixture. Table 1 compares the composition of the mixtures obtained from deconvolution of the phosphores-

cence spectrum (α) to that derived from the molar absorptivity (Wong and Eftink 1998) of the components. We note that phosphorescence overestimates the analog content by a small percentage in the case of 7-ATrp and 5-OHTrp, whereas it underestimates it up to 6% in the case of 5-FTrp (Table 1). In all cases, the discrepancies are within the experimental error of the preparation of the mixtures, and these experiments confirm the validity of phosphorescence for the determination of the analog incorporation efficiency in protein samples. The low ϕ_p^{An} of 7-ATrp (0.036) and the large difference in spectrum between Trp and 7-ATrp make it relatively easy to detect low percentages of Trp in the mixture. Spectral differences are also significant between Trp and 5-OHTrp. This approach is the least sensitive for

Figure 2. Low-temperature phosphorescence spectra of W30 and W30 alloproteins in 1,2 propylene glasses at 175 K. (A) Spectrum of W30. (B) Spectrum of 5-FTrp-containing W30 (solid) and the resulting fit using Equation 2 (dotted line). (C) Idem for 5-OHTrp-containing W30. (D) Idem for 7-ATrp-containing W30.

Figure 3. Low-temperature phosphorescence spectra of NATA and Trp analogs in 1,2 propylene glasses at 175 K. (A) Normalized spectra of NATA (solid line), 5-FTrp (dashed line), 5-OHTrp (dotted line) and 7-ATrp (dashed and dotted line). (B) Spectrum of a 22:78 NATA/5-FTrp mixture (solid line) and the fitted spectrum (dotted line). (C) Spectrum of a 19:81 NATA/5-OHTrp mixture (solid line) and the fitted spectrum (dotted line). (D) Spectrum of a 20:80 NATA/7-ATrp mixture (solid line) and the fitted spectrum (dotted line).

5-FTrp because ϕ_p^{An} is relative large ($\phi_p^{An} = 0.60$) and spectral differences are modest.

The procedure based on the phosphorescence properties of the free chromophores cannot be applied directly to the analysis of Trp/Trp analog emission in proteins because their phosphorescence may be modified, in both spectrum and quantum yield, by the protein environment. Therefore, to apply Equation 1, one requires knowledge of spectrum and quantum yield of both Trp and analog in the protein. Unfortunately, until the degree of incorporation is established, this information is not available for the analog form

of the protein. In principle, the analog spectrum of 7-ATrp and 5-OHTrp, free from any Trp contribution, can be obtained by selective excitation of the analog on the red edge, $\lambda_{ex} = 310$ nm, of the absorption spectrum. In such a case, its intensity contribution at $\lambda_{ex} = 295$ nm, at which both Trp and analog protein components absorb, can be readily estimated from the change in absorbance between the two wavelengths. For EII^{mtl}, however, the analog spectrum obtained on red edge excitation of the alloprotein is poorly characterized as, in the same spectral range, the phosphorescence emission from other chromophores, presumably ionized tyrosine, was found to be at least as intense. For that reason, an alternative method was adopted to Equation 1, based essentially on spectral decomposition, that does not require knowledge of the analog quantum yield in the protein. Equation 2 evaluates the fractional intensity (f_w) of the Trp component in the total alloprotein phosphorescence by fitting the latter in terms of the free analog spectrum and that of the parent Trp protein sample. The fraction α of Trp-containing proteins in the sample, hence the analog incorporation efficiency ($1-\alpha$), is then determined from the ratio between the Trp intensity of the alloprotein sample and the intensity measured for the parent Trp protein sample at the same concentration. Equation 1 is modified to yield f_w and α as follows:

$$\frac{I_p(\lambda)}{\int I_p(\lambda)} = f_w \times \frac{I_p^W(\lambda)}{\int I_p^W(\lambda)} + (1 - f_w) \times \frac{I_p^{An}(\lambda + x)}{\int I_p^{An}(\lambda)} \quad (2)$$

$$\alpha = f_w \times \int I_p(\lambda) / \int I_p^W(\lambda) \quad (3)$$

where $I_p(\lambda)$, $I_p^W(\lambda)$, and $I_p^{An}(\lambda + x)$ represent the spectra of the alloprotein, the Trp-containing protein and the free Trp analog shifted by x nm, respectively. $\int I_p(\lambda)$, $\int I_p^W(\lambda)$, and $\int I_p^{An}(\lambda)$ are the corresponding areas of these spectra. An extra fitting parameter (x) was introduced into Equation 2 to account for the wavelength shift in the analog spectrum caused by the protein environment.

The other significant spectral alteration is the sharpening of the 0,0 vibronic band caused by a generally more homo-

Table 1. Percentage of Trp analog in mixtures of NATA and Trp analogs (in duplo) based on absorption spectroscopy and spectral deconvolution of low temperature phosphorescence spectra

Trp analog	Analog in mixture (%)	Fitting results (%)	Δ (%)
5-FTrp	78	77	-1
5-FTrp	98	92	-6
5-OHTrp	81	83	2
5-OHTrp	95	98	3
7-ATrp	80	84	4
7-ATrp	95	96	1

geneous solvation site inside the protein, relative to the aqueous phase, a feature that is responsible for a generally poorer fit in this spectral region. Examples of spectral fitting, using Equation 2, are presented for the W30 alloproteins in Figure 2, B through D. The fitting and the analog incorporation efficiencies obtained with this procedure are given in Table 2. By simple visual inspection of the fits, it is evident that deviations are largest in correspondence of the 0,0 vibrational band, particularly for 5-FTrp and 7-ATrp analogs. In the case of red-shifted 5-OHTrp and 7-ATrp (Fig. 2C,D) the lack of intensity in correspondence of the 0-0 band position of Trp containing W30 (Fig. 3A) is already indicative of a very high analog incorporation. The results confirm a percentage of incorporation of $\geq 95\%$ with each analog (Table 2). As pointed out above, the accuracy of the determination is intrinsically smaller with the 5-FTrp analog. This problem is made worse with W30 because the spectral differences between W30 and 5-FTrp-containing W30 inside the protein are even less than for the free amino acids in solution.

To corroborate this experimental approach for the quantitative estimation of 5-FTrp in protein samples, we have extended the analysis to another single-Trp-containing EII^{mtl} mutant, W97. Under identical growth conditions, comparable incorporation efficiency is expected also for any other mutant, unless the alloprotein is unstable and results in lower expression levels, but this was not the case for W97. The low-temperature spectrum of W97 is quite well resolved (Fig. 4A), better than for the free chromophore (Fig. 3A), W30 (Fig. 2A), or other single-Trp mutants of EII^{mtl} (Broos et al. 2000), the sharp 0-0 vibrational band making detection of a low percentage of Trp in the Trp analog-containing W97 samples even more sensitive. The spectrum of 5-FTrp-containing W97 is presented in Figure 4B together with the results of spectral deconvolution. Again, the results (Table 2) indicate that incorporation is practically complete, a finding in agreement with that of W30. Lastly, we note that there are quite large differences between the W30 and W97 alloprotein spectra (Figs. 2A,B, 4A,B), indicating a particular sensitivity of 5-FTrp phosphorescence to the change in microenvironment.

Table 2. Percentage of Trp analog in EII^{mtl} alloproteins based on spectral deconvolution of low temperature phosphorescence spectra

Alloprotein	Trp analog (%)
5-FTrp W30	95
5-OHTrp W30	98
7-ATrp W30	99
5-FTrp W30 ^a	92
7-ATrp W30 ^a	87
5-FTrp W97	99

^a Indole analog was used.

Figure 4. Low-temperature phosphorescence spectra of W97 and 5-FTrp-containing W97 in 1,2 propylene glasses at 175 K. (A) Spectrum of W97. (B) Spectrum of 5-FTrp-containing W97 (solid line) and the resulting fit using Equation 2 (dotted line).

Incorporation of indole analogs into W30

L-Trp can be synthesized from indole and L-serine by the enzyme Trp indole-lyase (tryptophanase, EC 4.1.99.1), a pyridoxal phosphate-dependent enzyme (Lee and Phillips 1995). The biological function of this enzyme is to degrade Trp to indole, pyruvate, and ammonia, and the equilibrium of this reaction is toward degradation (Lee and Phillips 1995). The substrate specificity of tryptophanase is known to be quite broad because the enzyme accepts Trp analogs bearing a fluoro-, chloro-, or methyl-substituted indole (Lee and Phillips 1995). Tryptophanase is only expressed in the presence of an inducer of the *tna* operon, controlling expression of the tryptophanase enzyme. The natural inducer is L-Trp but also Trp derivatives such as 1-, 4-, 5-, or 6-methylTrp can induce this operon. Only 1-methyltryptophan (1-MTrp) is not a substrate of tryptophanase or Trp t-RNA acylase and can thus not be biosynthetically incorporated into a protein. This makes 1-MTrp an attractive inducer in experiments in which the biosynthetic incorporation of indole analogs is investigated. Trp auxotrophs could be grown on indole as precursor of L-Trp when the

appropriate inducer was present (Edwards and Yudkin 1984; Yanofsky et al. 1991).

Initial experiments were performed to find conditions for growing *E. coli* M5219 Trp auxotroph in a minimal medium, containing indole or an indole analog. Inoculation of an overnight culture of M5219 in Luria Broth (LB) medium into M9 medium with only 1 mM indole and 1 mM L-serine did not result in cell growth. As expected, cells grew in the presence of 1 mM L-Trp. When besides indole and serine also 0.1 mM L-1-MTrp was present in the growth medium, cells grew to almost the same OD as in the presence of Trp. This result indicates that the biosynthesis of L-Trp from indole is not limiting the growth of M5219 under these conditions. To investigate the incorporation of Trp analogs via their indole precursors, the protocol for the biosynthetic incorporation of Trp analogs was slightly modified (see Materials and Methods). The incorporation of 5-hydroxyindole (5-OHindole), 7-azaindole, 5-fluoroindole (5-Flindole), and 4-fluoroindole (4-Flindole) in W30 was investigated.

Incorporation of 5-OHindole

W30 was not expressed when 5-OHindole was present in the medium during induction. This result indicates that 5-OHTrp is not synthesized efficiently under the used growth conditions.

Incorporation of 7-azaindole

The presence of 7-azaindole in the medium resulted in expression of W30. The excitation fluorescence spectrum of the purified W30 alloprotein was red-shifted as was that of W30, expressed in the presence of Trp, although not as red-shifted as was found for W30, expressed in the presence of 7-ATrp (data not shown). In the phosphorescence spectrum, the 0-0 vibronic band of Trp at 411 nm is visible (Fig. 5A). Quantification of the incorporation efficiency via spectral fitting of the low-temperature phosphorescence spectrum, using W30 and free 7-ATrp as reference spectra, yielded an incorporation efficiency of 87% (Table 2).

Incorporation of 5-Flindole

W30 was overexpressed when the medium was supplemented with 5-Flindole. The phosphorescence spectra of the W30 alloprotein expressed in the presence of 5-FTrp or 5-Flindole were essentially the same (Fig. 5B). Spectral deconvolution of the phosphorescence spectrum of W30 obtained with 5-Flindole in the growth medium revealed that the 5-FTrp incorporation efficiency was 92%.

Incorporation of 4-Flindole

Also the presence of 4-Flindole in the growth medium resulted in overexpression of W30. The fluorescence excitation spectrum of the purified W30 alloprotein was blue-shifted with respect to the W30 spectrum, and the typical

Figure 5. Low-temperature phosphorescence spectra of W30 alloprotein in 1,2 propylene glasses at 175 K. (A) Spectrum of 7-ATrp-containing W30 with 7-azaindole present in the growth medium (solid line) and the resulting fit using Equation 2 (dotted line). (B) Spectra of W30-containing 5-FTrp, introduced into the protein via 5-FTrp (solid line) or via 5-Flindole (dotted line).

shoulder of Trp ~295 nm was not visible. Quantification of the incorporation efficiency via spectral deconvolution of the phosphorescence spectrum was not possible because of the blue-shifted absorption spectrum of 4-FTrp (Bronskill and Wong 1988). Efficient excitation of 4-FTrp is only possible at ≤ 290 nm wavelengths, but at this wavelength the 11 tyrosine residues present in W30 were excited and their phosphorescence emission dominated the low-temperature phosphorescence spectrum. 4-FTrp is not fluorescent (Bronskill and Wong 1988; Hott and Borkman 1989), and an emission spectrum of the alloprotein, comparable to Trpless EII^{mtl}, is expected in the case of a high incorporation efficiency. The emission spectra of 4-FluoroTrp-containing W30 show a maximum at 305 nm, typical for tyrosine, and the spectrum is essentially similar to that of Trp-less EII^{mtl} (maximum at 304–305 nm; Fig. 6; Swaving Dijkstra et al. 1996a). This proves that 4-Flindole has been incorporated in this sample with very high efficiency.

Figure 6. Normalized fluorescence emission spectra of Trp-less EII^{mtl} (solid line) and 4-FTrp-containing W30 (dotted line). The excitation wavelength was at 290 nm. Bandwidths were set at 4 nm.

Discussion

Biosynthetic incorporation of a Trp analog into a protein is relatively easy, once a plasmid-borne bacterial expression system has been developed (Ross et al. 1997). In fact, a few extra centrifugation steps are needed to remove Trp from the growth medium and replace it by the Trp analog before induction. A Trp auxotroph is needed as expression host, and a number of different *E. coli* auxotrophic strains have been created to achieve this goal. A correlation between the Trp auxotrophic strains and the efficiency of generating a Trp alloprotein has not been reported. What seems to be most critical is a promoter system preventing expression of the target protein when growing the cells in the Trp-containing medium. Good results have been obtained with the *tac* (Ross et al. 1997) and the *pBAD* (Mohammadi et al. 2001) promoters. Depleting the cells of Trp before induction is also of importance for a high analog incorporation efficiency. The few documented studies in which 5-OHTrp, 7-ATrp, and fluoroTrp were introduced at the same protein residue position indicated that the incorporation efficiency was dependent on the analog structure (Wong and Eftink 1998; Mohammadi et al. 2001). In this article, we report on the use of the heat-inducible λP_R promoter system to generate integral membrane alloproteins. Originally, the EII^{mtl} gene was overexpressed in *E. coli* with the pMa plasmide, containing the λP_R promoter and the gene for the heat-labile cI₈₅₇ repressor protein (van Weeghel et al. 1990). This plasmid system was also used to transform the M5219 Trp auxotroph, a strain with a chromosomal copy of the gene for the cI₈₅₇ repressor protein. The very high to complete Trp analog incorporation efficiency in W30 and W97 is indicative for a tightly regulated promoter system. The presence of the dual expression routes of the repressor protein in the

pMa/M5219 system might be responsible for this. The EII^{mtl} expression levels are as high in M5219 as in the *E. coli* LGS strain (Grisafi et al. 1989), a non-Trp auxotroph strain, routinely used for the expression of EII^{mtl} (Swaving Dijkstra et al. 1996a).

An important aspect of alloprotein generation relates to the ability to estimate the analog incorporation efficiency, and several procedures have been reported for Trp analogs. The most straightforward approach is deconvolution of the alloprotein absorption spectrum, using the spectra of the free Trp and Trp analog, with protecting groups at the N and C terminus, as parent spectra (Waxman et al. 1993). The spectra are recorded under denaturing conditions in 6 M guanidinium chloride, to eliminate spectral changes induced by the protein structure. Two different methods have been reported (Soumilion et al. 1995; Budisa et al. 2001; Senear et al. 2002). In one method, the alloprotein was denatured and separated by reverse-phase HPLC. The replacement of Trp by an analog can cause a different retention time, and the incorporation efficiency was estimated from the peak areas. The second method makes use of mass spectrometry, an approach useful for most analogs, except for 7-ATrp because its mass differs only 1 D from Trp.

We found these methods not suitable for the enzyme system we are investigating, the mannitol transporter, EII^{mtl}, from *E. coli*. This protein of 637 amino acids is an integral membrane protein, functional as a dimer, and responsible for the uptake and phosphorylation of mannitol (Robillard and Broos 1999). Integral membrane proteins have some properties in common, which makes them difficult to analyze for their Trp analog content with the current methodologies available. Generally, a significant portion of the structure consists of transmembrane helices making membrane proteins-resistant toward complete unfolding using denaturants. The solubility of purified protein, solubilized by detergent, is limited, and the large protein-micelle complexes, >300 kD in the case of EII^{mtl} (Lolkema et al. 1993), cause light scattering. Altogether these factors prohibit accurate analysis of the Trp analog-containing membrane protein via absorption spectroscopy. The presence of detergent severely limits the analysis of membrane proteins by using mass spectrometry. Mass spectrometry analysis of membrane proteins after fragmentation with a protease or CNBr typically results in partial coverage of the protein sequence because resistance toward unfolding prohibits complete digestion and some digestion products are too hydrophobic and aggregate. After significant effort, up to 83% of the sequence of membrane-embedded domain of EII^{mtl} could be detected with mass spectrometry (van Montfort et al. 2002).

A different method was therefore needed to estimate the Trp analog incorporation efficiency. Here, we describe an approach based on the analysis of the low-temperature phosphorescence emission of alloprotein samples as a linear

combination of the Trp and Trp analog contribution. There are distinct advantages of using phosphorescence in place of the corresponding fluorescence signal. The spectrum of the delayed emission is far better resolved and exhibits a characteristic vibrational structure specific of each chromophore, a feature that, together with the red shift of the spectrum, permits a more sensitive discrimination between Trp and analog signals. Further, undesired contributions to the overall intensity by tyrosine/tyrosinate can be readily subtracted in phosphorescence because, contrary to fluorescence, their spectrum is well resolved from that of Trp and of Trp analogs. The delayed emission does not suffer the limitations imposed by scattering of the excitation and can therefore be monitored in practically any medium, including proteins embedded in micelles or membranes. Finally, low temperature (<180 K) and the glass state are conditions that drastically lower both intramolecular and diffusion-controlled (dynamic) quenching reactions, leading to large and reproducible luminescence yields. Naturally, the sensitivity of the method increases with difference in spectrum and quantum yield between Trp and Trp analog; a low quantum yield of the analog enhances the ability to detect low Trp contents. On these grounds, the accuracy of the determination is expected to be highest for 7-ATrp and smallest for 5-FTrp. The main disadvantage of the technique is the need to work at low temperature with instrumentation not commonly available.

Feeding of the M5219 auxotroph with the Trp analogs 7-ATrp, 5-OHTrp, or 5-FTrp resulted in very high incorporation efficiencies in EII^{mtl} (Table 2). These results are also supported by the fluorescence excitation spectra (Fig. 1). In the case of the 7-ATrp-W30 protein, an additional confirmation that Trp is not present in this sample was obtained from time-resolved phosphorescence decays in buffer at 0°C. In fluid media, free or protein-embedded 7-ATrp is weakly phosphorescent with an average lifetime, $\tau_{av} < 1$ msec (Cioni et al. 1998). Excitation of 7-ATrp-containing W30 at 293 or 314 nm gave a similar decay, with a τ_{av} of 0.9 msec, the longest component being 1.5 msec. In these decays, there is no trace of Trp components with the characteristic τ_{av} of 18.4 msec obtained for W30 under these conditions (Broos et al. 2000). This, and the fact that some trace protein impurities could be seen when the alloproteins were analyzed by acrylamide gel electrophoresis and Coomassie blue staining, indicates that the efficiency to incorporate Trp analogs into EII^{mtl} as compiled in Table 2 is probably absolute.

Indole analog incorporation

Only a limited number of Trp analogs are commercially available. Except for 5-OHTrp, all these analogs are sold as racemic mixtures. The cost can be high, and 4-FTrp is very expensive. A large number of indole derivatives are com-

mercially available and are usually much cheaper than the corresponding Trp analog. In this article, we demonstrate that Trp analogs can be efficiently incorporated from indole as starting material. The *in vivo* synthesis is catalyzed by tryptophanase, which couples indole with L-serine and yields L-Trp. Feeding the M5219 auxotroph with 5-OHindole did not result in expression of W30, a result indicating that 5-OHTrp is not efficiently synthesized *in vivo*. Feeding with 4-Indole, 5-Indole, or 7-azaindole resulted in expression of W30 at expression levels comparable to the expression in the presence of the Trp analog. Analysis of the spectra of the purified alloproteins proved that incorporation efficiencies could be very high. With 5-Indole, an incorporation efficiency of 92% was measured, slightly less than for 5-FTrp (95%) and a difference within the experimental error of the fitting procedure. Incorporation of 7-azaindole was somewhat less efficient, with an incorporation efficiency of 87% compared with 99% for 7-ATrp. The emission spectrum of W30 expressed in the presence of 4-Indole was found to match the emission spectrum of Trp-less protein (Fig. 6). Because 4-FTrp is not fluorescent, this result is expected if 4-FTrp incorporation is complete. Apart from commercial availability and economical reasons, the fact that no D-Trp analog is introduced to the growth medium might be another reason to use indole analogs. D-amino acids, including D-Trp, can be toxic and arrest the growth of an organism. Recently, it has been shown that the toxicity of D-Trp in *E. coli* is related to its incorporation into proteins (Soutourina et al. 2000). *In vitro* studies pointed out that the stereospecificity of Trp-tRNA synthetase is not absolute, and D-Trp-tRNA^{Trp} is formed, albeit at a much lower efficiency than is L-Trp. A proofreading system constituting the aminoacyl-tRNA deacylase enzyme, exhibiting a high substrate specificity toward the D-aminoacyl-tRNA, efficiently eliminates D-Trp-tRNA^{Trp}. This prevents the translation of D-Trp. Knock-out strains lacking this deacylase enzyme are very susceptible for the presence of D-amino acids, and millimolar concentrations of D-Trp efficiently abolished growth (Soutourina et al. 2000). The stereospecificity of the tRNA acylase and deacylase enzyme toward Trp analogs is not known. Investigation of the stereospecificity of the tRNA acylase and deacylase enzyme is needed to clarify if D-Trp analogs can end up in the target protein.

In conclusion, the work presented in this article demonstrates that different Trp analogs can be very efficiently incorporated into a membrane protein when overexpression is under control of the heat-inducible λP_R promoter and M5219 is used as expression host. The protocols both to produce recombinant membrane-embedded Trp alloproteins and to estimate the Trp analog content open new routes to characterize this important class of proteins by using Trp analogs. We are currently exploring the fluorescence and phosphorescence properties of the EII^{mtl} alloproteins presented in this article at ambient temperatures.

Materials and methods

Chemicals and reagents

5-FTrp, 5-OHTrp, 7-ATrp, 5-OHindole, and NATA were from Sigma. 4-Indole, 5-Indole, 7-azaindole and 1-MTrp were from Aldrich. 1,2 propylene glycol was from Across Organics. C₁₀E₅ was from Kwant High Vacuum Oil Recycling and Synthesis. Fluorescent impurities were removed as described (Swaving Dijkstra et al. 1996b).

Plasmids and bacterial strains

Plasmids of the single-Trp mutants of EII^{mtl} (W30, W97) containing the λP_R promoter and the cI₈₅₇ temperature-sensitive repressor (Swaving Dijkstra et al. 1996a) were engineered to obtain EII^{mtl} with an N-terminal thrombin-cleavable *his*-tag. Construction of the plasmid specifying the N-terminal his-tagged EII^{mtl} will be published elsewhere.

The W97 mutant was created, starting from the plasmid containing the Trp-less EII^{mtl} gene in a similar way by using the 5' CATAGATCCGAGCCACATCG-3' primer. This primer also introduced an additional *Xho*II restriction site. The *E. coli* bacterial strain LGS-322 (Grisafi et al. 1989) in LB medium plus 100 μ g/L ampicillin was used for the expression of EII^{mtl} mutants containing Trp. The *E. coli* Trp auxotroph M5219 (λ cI₈₅₇ lysogen, *Trp*_{am}; Remaut et al. 1981), obtained from BCCM/LMBP was used for the incorporation of Trp analogs.

Growth and induction of bacteria

A 20 mL overnight culture of M5219 in LB medium plus 100 μ g/L ampicillin was added to 1L M9 minimal medium (Sambrook et al. 1989) supplemented with 0.5% glucose, 0.1% thiamine, 0.1 mM CaCl₂, 1 mM MgCl₂, 0.01 mM FeSO₄, trace elements (Favre-Bulle et al. 1993), 100 μ g/mL ampicillin, and 1 mM L-Trp. The culture was placed in a 5-L Erlenmeyer flask and shaken at 250 rpm at 30°C until OD₆₀₀ = 0.6 to 0.7. Cells were collected by centrifugation, washed with M9, and resuspended in the same volume of medium, without L-Trp. Shaking was continued for 30 min at 30°C, followed by the introduction of 1 mM DL-Trp analog or 0.5 mM L-5-OHTrp, dissolved in dilute NaOH. After 15 min at 30°C, the temperature was raised in ~2 h to 42°C and incubation was continued for 0.5 h. Cells were collected by centrifugation and washed with 25 mM Tris-HCl buffer (pH 7.5) and 5 mM DTT. For the incorporation of Trp analogs via their indole analogs, 0.1 mM 1-methylTrp was added to the resuspended and washed cells, following the same procedure as outlined above. After shaking the washed cells for 30 min at 30°C, 1 mM indole and 1 mM L-serine were added, and shaking was continued for 15 min, followed by increasing the temperature to 42°C as described. Preparation of inside-out membrane vesicles (Broos et al. 1999), purification of the *his*-tagged enzymes (Broos et al. 2000), activity measurements of EII^{mtl} (Robillard and Blaauw 1987), and EII^{mtl} concentration determinations (Brouwer et al. 1982) were performed as described.

Fluorescence measurements

Fluorescence spectra were recorded on an SLM-Aminco SPF-500 fluorometer at room temperature. The excitation and emission band passes were set at 2 and 5 nm, respectively. The buffer used was 20 mM Tris-HCl (pH 8.4), 0.25 M NaCl, 0.25% C₁₀E₅, and 1

mM reduced glutathione. All spectra were corrected for fluorescence from the buffer.

Phosphorescence measurements

A conventional homemade instrument was used for all phosphorescence measurements using low-temperature (175 K) glasses. The excitation provided by a Cermox xenon lamp (LX 150 uv; ILC Technology) was selected by a 0.25-m grating monochromator (Jobin-Yvon, H25; 5-nm bandpass), and the emission dispersed by a 0.25-m grating monochromator (Jobin-Yvon, H25; 1.5-nm bandpass) was detected with an EMI 9635QB photomultiplier. The buffer used was the same as for the fluorescence measurements. EII^{mtl} solution (4 to 15 μ M) was mixed with 1.03 part 1,2 propylene glycol (PG; w/w), followed by cooling down the sample in the cuvette holder to 175 K. Excitation was at 295 nm.

Fitting of the phosphorescence spectra

Before data analysis of the alloprotein spectra, the non-Trp emission (solvent, tyrosine/tyrosinate contributions) was subtracted from all phosphorescence spectra by using the emission of the Trpless EII^{mtl} protein. Spectral deconvolution was achieved by means of a least-squares algorithm implemented in the program Solver of Excell (Microsoft).

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