Structural and functional studies on EII from Escherichia Coli, using fluorescence and FTIR spectroscopy.
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The enzyme responsible for mannitol transport in *Escherichia coli*, EII\(^{\text{ad}}\), has been studied with two techniques: fluorescence spectroscopy and Fourier transform infrared (FTIR) spectroscopy. This enzyme belongs to the class of phosphoenolpyruvate-dependent phosphotransferase systems, which couple phosphorylation and transport of carbohydrates over the membrane. Phosphoenolpyruvate-dependent phosphotransferase systems are comprised of carbohydrate-specific membrane-bound transporters and several cytoplasmic proteins responsible for phosphoryl group transfer from phosphoenolpyruvate to the carbohydrate transported.

This research focuses on EII\(^{\text{ad}}\). This is a three domain enzyme from the cytoplasmic membrane of *E. coli*. Two cytoplasmic domains, IIA\(^{\text{ad}}\) and IIB\(^{\text{ad}}\), are involved in phosphoryl group transfer, while the third domain, IIC\(^{\text{ad}}\), which is embedded in the cytoplasmic membrane, is responsible for mannitol binding and transport.

The three-dimensional structures of the cytoplasmic domains, IIA\(^{\text{ad}}\) and IIB\(^{\text{ad}}\), are currently being solved with X-ray crystallography and NMR spectroscopy. These techniques are not suited for revealing the structure of IIC\(^{\text{ad}}\). With fluorescence and FTIR spectroscopy it is possible to derive structural information on this domain, which is necessary to gain insight in the complex molecular processes occurring during mannitol transport over the membrane.

The fluorescence characteristics of tryptophan can be used to study the structure and dynamics of a protein. Most information concerns local features in the vicinity of the tryptophan residue and processes which lead to measurable changes in the fluorescent characteristics of the tryptophan.

FTIR spectroscopy does not depend on specific chromophores, but relies on the absorbance of all bonds. The frequency of some characteristic absorbance bands, which represent vibronic transitions of peptide groups, is related to the secondary structure elements present in the protein. Furthermore, it is possible to monitor structural changes of a protein as a function of light, heat, pH or other parameter, using difference spectroscopy or hydrogen-deuterium exchange.

In chapter 1 membrane proteins, PTS, fluorescence spectroscopy and FTIR spectroscopy are discussed.

Chapter 2 describes a procedure developed to purify EII\(^{\text{ad}}\) and mutants of EII\(^{\text{ad}}\) free of fluorescent impurities. A mutant without tryptophan residues was essential for the development of this procedure, which relies on use of a highly pure detergent and avoiding contact with plastics and rubbers of any kind during all stages in the purification procedure. In this functionally active mutant the four natural tryptophans of EII\(^{\text{ad}}\) at positions 30, 42, 109 and 117 are replaced by phenylalanines. Fluorescence spectra of this mutant indicate that all buffers tryptophans accumulate, tryptophans is impossible to interpret tryptophan proteins. Tryptophans show.

In chapter 3 single-tryptophan of the natural tryptophans phosphorylated tryptophan tryptophan fluorescence.

Chapter 4 contains an increase fluorescence tryptophan properties.
The enzyme from the E. coli, EIIn<sup>+</sup>, has been shown to transform infrared spectroscopy. These enzymes are involved in the mannanophosphorylase reaction during mannitol phosphorylation. The presence of fluorescent impurities in EIIn<sup>+</sup>-samples was essential for the study of the structure of these enzymes. The fluorescent impurities were not distinguishable from tryptophan on the basis of their fluorescence emission spectra or fluorescence lifetimes. It is impossible, without using the guidelines from the procedure mentioned above, to safely interpret tryptophan fluorescence spectra of EIIn<sup>+</sup> and presumably other membrane proteins. This finding implies that other tryptophan-fluorescence studies on membrane proteins should be treated with some caution.

In chapter 3 the results of different steady-state fluorescence experiments on six single-tryptophan mutants of EIIn<sup>+</sup> are discussed. The tryptophan residue is located in one of the natural positions 30, 42, 109 or 117, or in a special position (320 or 384). The other tryptophans are replaced by phenylalanines, without destroying the mannitol phosphorylation activity. An exception is the W384-mutant, in which the second phosphorylation site cysteine 384 in IIB<sup>+</sup> is replaced by tryptophan. The various single-tryptophan mutants, purified using the procedure of chapter 2, show differences in fluorescence emission maxima. Emission maxima at relatively low wavelengths for tryptophans 30 and 320 indicate a hydrophobic environment, whereas tryptophans 42, 109, 117 and 384 are somewhat more hydrophilic. This is the first time that clear differences in the fluorescence emission maxima of different single-tryptophan mutants of a purified membrane protein have been reported. Results of iodide quenching experiments indicate a more buried position for tryptophans 30, 42 and 320 in the EIIn<sup>+</sup>-structure than tryptophans 109 and 117. Both the frequency of the fluorescence emission maxima and the iodide quenching support the predicted topology of EIIn<sup>+</sup> (Sugiyama et al., 1991), with positions 30, 42 and 320 in transmembrane α-helices and positions 109 and 117 in a cytoplasmic loop. Tryptophan 384 in IIB<sup>+</sup>, at the position of the second phosphorylation site of EIIn<sup>+</sup>, is almost non-fluorescent at physiological pH. This could be due to a strong quenching by a nearby carboxylate or tyrosinate residue in the B-domain (AB et al., in press). Lowering of the pH results in unfolding of IIB<sup>+</sup> (Meijberg et al., 1996) and a strong increase in fluorescence intensity of this tryptophan. Whether these residues are critical for the mechanism of phosphoryl group transfer to mannitol is not yet known.

Phosphorylation of EIIn<sup>+</sup> does not result in mentionable changes in the fluorescence of the single-tryptophan mutants or of wild-type EIIn<sup>+</sup>. Mannitol binding, however, induces an increase in fluorescence intensity (Wood, 1988) mainly due to an increase in fluorescence intensity of tryptophan 30, as can be seen using the different single-tryptophan mutants. This indicates that W30 is able to sense mannitol binding.

Chapter 4 presents time-resolved fluorescence experiments on the various single-tryptophan mutants. These experiments offer detailed information on the fluorescent properties and the different motions of the tryptophans. The time-dependent decrease in
fluorescence intensity can be adequately described by a distribution of several fluorescence lifetimes ($\tau$), each probably representing the fluorescence lifetime of a rotameric state of the tryptophan. Different average fluorescence lifetimes $<\tau>$ for each tryptophan were observed. The decrease in fluorescence anisotropy for each tryptophan can be described by a distribution of different rotation correlation times ($\phi$), which correspond with different rotational motions of the tryptophan during its fluorescence lifetime. This can be internal motions of the tryptophan, like rotations around the C$_\alpha$-C$_\beta$ axis, or rotation of the entire protein or a portion of the structure. The anisotropy decay differs for the various tryptophans indicating differences in rotational motions due to differences in local structure surrounding the tryptophans. The limited contribution of motions represented by relative short correlation times $\phi$ of 0.3 - 4.7 ns to the total anisotropy decay, implies that all tryptophans are highly immobilized in the protein. These limited internal mobilities can be best seen from the order parameter ($S$), which is related to the angle of displacement ($\psi$) responsible for the rapid internal motion. The order parameter, $S$, varies from 0.90 to 0.97 for the tryptophans, which means they are highly immobilized and largely rotate together with the entire enzyme ($\phi$ non-resolvable) or a portion of the enzyme. Only for tryptophan 30 a $\phi$ of about 32 ns is resolved repeatedly, which corresponds with the motion of a complex of about 40 kDa. This could represent motion of a monomeric unit of IIC$^{\text{III}}$ independent from the rest of EII$^{\text{III}}$. Though the other tryptophans are also located in this domain and thus also should sense the motion of this domain, it is possible that this is not expressed in the anisotropy decay due to an unfavourable orientation of these tryptophans with respect to the rotational axis of this domain. Furthermore it is possible that the long lifetime component in W30, and the limited internal mobility of this tryptophan ($S = 0.97$), both favourable conditions for the determination of high correlation times, also play a role.

Binding of mannitol and perseitol (a substrate analogue, which binds to EII$^{\text{III}}$, but cannot be phosphorylated) results in the disappearance or decrease of the short-lived components of the fluorescence decay of tryptophans 30, 42 and 109 and an increase in $<\tau>$ for W30 and W42. This indicates changes in the putative first membrane-spanning $\alpha$-helix in IIC$^{\text{III}}$ induced by substrate binding. This is a surprising result since all positions in IIC$^{\text{III}}$ which have thus far been identified as important in mannitol binding are located in a large cytoplasmic loop connecting putative membrane spanning $\alpha$-helices 4 and 5.

Phosphorylation of the single-tryptophan mutants, on histidine 554 in the A domain and cysteine 384 in the B domain, results in an increase in the average fluorescence lifetimes of all tryptophans. This increase is most pronounced for W30 and W109, where the short fluorescence lifetime disappears analogous to the effect of mannitol binding. This is spectroscopic evidence for the coupling between the phosphorylation state of the cytoplasmic domains of EII$^{\text{III}}$ and the membrane-bound domain. This coupling has been determined earlier with kinetic experiments (Lolkema et al., 1991): phosphorylation results in a 1000 fold increase in catalytic rate.

The effects of different tryptophan mutants in a decrease in mobility of all tryptophans, some mutants show, e.g., W109, for which $S = 0.97$, an increase in the $\phi$ with respect to the correlation times in IIC$^{\text{III}}$ and the present.

In chapter 5 IIC$^{\text{III}}$ and to study the construct is used, to study the proteins. An additional FTIR spectrum corresponds with the secondary structures. The $\alpha$-helical structure of IIIB$^{\text{III}}$ and IIIC$^{\text{III}}$ exist in the FTIR spectrophotometric spectra of the proteins of EII$^{\text{III}}$.

The FTIR Phosphorylation of intensity changes between helices 4 and 5 in an equimolar solution IIIB$^{\text{III}}$ and IIIC$^{\text{III}}$ exist dependent coupling sheet structure of EII$^{\text{III}}$. The results have been recognized to be recognized with the structure between helix 4 and 5 in the part of EII$^{\text{III}}$. This couple is independent EII$^{\text{III}}$. The results.
of several fluorescence decay times for a rotameric state of each tryptophan were determined. This can be described by internal rotation of the entire tryptophan residues. For the various tryptophans represented by relative mobilities, it was found that all tryptophans largely rotate together. Only for tryptophan \( W_{109} \) is no rotation observed. This is also located in the connecting structure between helices 4 and 5. In the \( \alpha \)-helical structure, the connecting structure between helices 4 and 5 appears to contain a certain amount of \( \beta \)-sheet structure.

The FTIR spectra of IIC representable structure elements in the membrane-embedded C domain. The results of the FTIR experiments are the first spectroscopic information, in a 1000 fold increase in the rate of mannitol transport catalysed by EII\textsuperscript{nd}. The effects of mannitol binding and phosphorylation on the anisotropy decay of the different tryptophans was in most cases rather small. Mannitol binding resulted for all mutants in a decrease in \( \alpha \) of 1 - 3\%, corresponding to a small increase in the internal mobility of all tryptophans. Phosphorylation induced changes in \( \alpha \) of similar magnitude, some mutants showing small decreases in \( \alpha \) and others small increases. An exception is \( W_{109} \), for which \( \alpha \) increased from 0.90 to 0.97. This corresponds to a decrease in the angular displacement of the rotation from 30\(^\circ\) to 16\(^\circ\). A different effect of phosphorylation is an increase in the 32 ns correlation time of \( W_{30} \), which possibly corresponds to rotation of the C domain, to 51 ns. This could indicate a changed orientation of this tryptophan with respect to the rotational axis of IIC\textsuperscript{nd}. Together with the absence of comparable correlation times in the other mutants this implies asymmetry in the micelle-embedded IIC\textsuperscript{md} and the presence of different rotational motions.

In chapter 5 FTIR spectroscopy is used to study secondary structure elements in IIC\textsuperscript{nd} and to study the coupling between IIC\textsuperscript{nd} and (P-)IIB\textsuperscript{nd}. For this purpose a IICB\textsuperscript{nd}-construct is used, which is fully active in the presence of IIA\textsuperscript{nd} and the other PTS components. This offers the possibility to monitor the influence of peretitol binding on the secondary structure elements of P-IICB\textsuperscript{nd}.

FTIR spectra of IIC show a strong absorption band at 1657 cm\(^{-1}\), which corresponds with the amide I absorption of \( \alpha \)-helical structure specific for membrane proteins. An additional band at 1630 cm\(^{-1}\) is evidence for the presence of \( \beta \)-sheet structure. The \( \alpha \)-helical structure can be explained by the presence of transmembrane \( \alpha \)-helices (Sugiyama et al., 1991). The \( \beta \)-sheet structure could be present in the cytoplasmic "loop" between helices 2 and 3 and/or between helices 4 and 5.

The FTIR spectra of IIC\textsuperscript{nd} are almost identical to the spectra of IIC\textsuperscript{md}. Phosphorylation of ICB\textsuperscript{nd}, both in the presence and absence of peretitol, leads to small intensity changes and shifts in the \( \beta \)-sheet region. Since these changes are not seen in equimolar solutions of IIC\textsuperscript{nd} and (P-)IIB\textsuperscript{nd}, in which only negligible amounts of (P-)IIB\textsuperscript{nd} exist under FTIR conditions, they have to be due to a phosphorylation-dependent coupling between the B and C domain in IICB\textsuperscript{nd}, resulting in changes in the \( \beta \)-sheet structure of the C domain. This finding and the finding that all residues, which have been recognized to be important for mannitol binding, transport and/or phosphorylation (Manayan et al., 1988; Weng et al., 1992; Boer et al., 1996), are located in the connecting structure between helices 4 and 5 in the C domain, suggest that the change occurs in this part of IIC\textsuperscript{nd}. This region, thus far indicated as "large cytoplasmic loop" between helices 4 and 5, possibly contains a certain amount of \( \beta \)-sheet structure.

Structural information has been derived for the membrane embedded C domain of EII\textsuperscript{nd}. The results of the fluorescence experiments are the first spectroscopic information,
which confirm the predicted topology of EII₅. Other important results are the observation that, upon mannitol binding, changes occur in the first transmembrane α-helix and the observation that the phosphorylation state of the cytoplasmic domains A and B affect the fluorescent properties of tryptophans at different positions in the C domain. Time-resolved fluorescence experiments indicate the presence of rotational motions of IICᵢᵣ independent from the rest of EIIᵢᵣ and asymmetry in this micelle-embedded domain. The FTIR results imply the presence of β-sheet structure in IICᵢᵣ in addition to α-helical structure. This β-sheet structure, which changes upon phosphorylation of the B domain, is presumably present in the cytoplasmic "loop" connecting α-helices 4 and 5 in IICᵢᵣ.

The information accumulated with these studies adds to a better understanding of the EII₅ structure and the conformational changes which occur in this enzyme during mannitol transport and phosphorylation.