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Lack of correspondence between the room-temperature phosphorescence decay-components and Trp residues in a series of Trp→Cys or Trp→Phe mutants of human carbonic anhydrase II

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Abstract The room temperature phosphorescence of native human carbonic anhydrase (CA), and several mutants of this enzyme has been investigated. In these mutants the seven tryptophan residues in the native protein have sequentially been replaced by cysteine or phenylalanine. All of the mutants as well as native CA show room-temperature tryptophan phosphorescence (RTP) spectra. Surprisingly, only small differences in RTP life-times are noticeable among these mutants, indicating that there is more than one tryptophan residue with similar phosphorescence decay kinetics in the protein. The present results illustrate the danger in attributing the room temperature phosphorescence of a multi-tryptophan protein to a particular residue based solely on an analysis of the protein structure.

Key words: Carbonic anhydrase; Room-temperature phosphorescence; Mutant protein; Tryptophan; Laser spectroscopy

1. Introduction

The enzyme carbonic anhydrase (CA) is found in most organisms [1]. The CA gene family contains at least 7 enzymatically active isozymes in higher eukaryotes. The gene structures and amino acid sequences have been determined from a number of species [2], the crystal structure has been investigated in detail [3], and the folding of several isozymes has been studied extensively. By using site directed mutagenesis, the single cysteine residue in isozyme II from human (HCA II) has been removed. A number of additional residues have been mutated as well; cysteine residues have been introduced in novel positions, and by reacting these residues with alkylating reagents during folding, the relative rates of structure formation of several parts of the molecule have been studied [4]. During these studies, it was found that when W97 was replaced with cysteine, this residue was partially protected from alkylation, even at concentrations of guanidine-HCl (GuHCl) as high as 8 M. Additional mutations that replace each of the remaining seven native tryptophan residues have been expressed, and the fluorescence properties of these mutant forms of HCA II have been investigated. It was found that two of the native tryptophan residues, W97 and W245, are the major fluorescence emitters, contributing 52% and 37%, respectively, of the total fluorescence intensity when the protein is excited at 295 nm [5].

A large number of proteins show tryptophan room- temperature phosphorescence (RTP) in the absence of oxygen [6]. Since the triplet state is populated via the photoexcited singlet state, a critical factor effecting the yield of RTP is the extent to which the fluorescent state is quenched prior to intersystem crossing. Furthermore, since there is a direct correlation between phosphorescence life-time and microviscosity at the site of emission [7], only those tryptophan which are located in the most constrained regions of the protein are expected to possess long (ms) phosphorescence decay times. Therefore in proteins containing more than one tryptophan the emission originating from solvent exposed tryptophans, typically with submillisecond life-times, is often obscured by the emission of rigidly disposed residues showing life-times up to two seconds. Single residue phosphorescence in multi-tryptophan containing proteins appears so far to be the rule rather than the exception (see references in [8]), and the residue responsible for the long-life-time RTP is often assigned based on the locations of the tryptophans in the protein structure [9]. If the RTP decay components can be assigned to specific residues, and especially in proteins where the RTP is originating from a single residue, this technique can serve as a very specific probe for the local structure around the emitting tryptophan. RTP has been used to investigate changes in the rigid core of the protein, both during unfolding in denaturants [10], as well as dilution-induced refolding [11].

Given the previous observations that W97 and W245 are the predominant fluorescing residues in HCA II, that the W97 is partly inaccessible to alkylation even under denaturing conditions and that this residue is completely shielded from the solvent [5], W97 is a likely candidate to show a long RTP life-time in this protein. To investigate the role of W97 in the RTP of HCA II, as well as to address the important question whether phosphorescence decay components in multi tryptophan proteins can be assigned to individual residues, we here report a study of the room temperature phosphorescence of HCA II mutants generated by sequentially removing each tryptophan residue in the enzyme, such that each mutant lacks one or two native tryptophan residues.

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2. Materials and methods

2.1 Expression of HCA II mutants in E. coli

Mutants of HCA II were produced by the method of Kunkel [12] in the pACA plasmid [13] and used to transfect *E. coli* BL21(DE3) [14]. The mutagenesis is described in more detail elsewhere [5].

2.2. Protein purification

E coli BL21(DE3) harboring the mutant plasmid was cultured in LB medium, supplemented with 0.5 mM $ZnSO_4$ and 50 μg/ml ampicillin at 23°C. When the culture reached an OD_{660} of 0.5, IPTG was added to 0.5 mM, and the cells were cultured for 6–12 h. The HCA II mutants were purified in one step on a affinity resin [15] (to prevent sample cross-contamination, chromatographic materials were freshly prepared for each mutant) and the purity was analyzed with SDS-PAGE. The protein concentrations were determined spectrophotometrically at 280 nm, and the extinction coefficients of the mutants were determined by the method of Gill and von Hippel as described elsewhere [5].

2.3. Phosphorescence measurements

To ensure that all proteins were examined under identical conditions, 50 mM Tris-SO_4, pH 7.5 buffer was added to the proteins and spun in a Centricon 100; this was repeated until the solution was exchanged by a factor of at least 10^6. The protein concentration was adjusted to 13 μM, and the phosphorescence was determined in protein solutions deoxygenated by extensive flushing of the cuvette with argon [16]. Phosphorescence was observed after exciting the sample with a pulse (ca. 8 ns) of light at 280 nm; an equal number of decays were collected form each mutant. Methods used for acquiring spectra, decays and subsequent analysis were as previously described [8].

3. Results and discussion

The phosphorescence decays of the pseudo wild type HCA II (C206S), and of the double mutants, where in addition to the C206S mutation, a second mutation was introduced that changes a tryptophan residue to cysteine or phenylalanine are shown in Fig. 1 (the phosphorescence decay of native HCA II obtained from Sigma Chemical Co. was also measured, and found to be very similar to the C206S pseudo wild type). The decay of the pseudo wild type clearly shows the presence of short and long life-time components. As discussed in the introduction, one would expect that these arise from distinct tryptophan residues, and that when these Trp residues are removed, the corresponding components of the decay would disappear. More specifically, based on previous experience with other proteins [8,17], we expected the longest decay component to be uniquely associated with the most buried, rigidly located Trp, i.e. W97. Surprisingly, only slight differences were found among the decay kinetics of the various mutants, and most striking is the presence of a long life-time component of ca. 9.3 ms in *each* of the mutants. In the past, protein RTP has invariably been assigned to tryptophan; however, the surprising similarity of decay kinetics in the mutants raise the possibility that other chromophores, most probably tyrosine, could be the source of long-lived emission. That this is not the case is clearly demonstrated by the fact that the observed steady state phosphorescence spectra shown in Fig. 2 can be unambiguously assigned to tryptophan [18]. Furthermore, time-resolved emission spectra obtained by selectively gating the short life-time components (0.1 μs–1 ms), or the long-lived components (1–50 ms) of the decay (data not shown) reveal that tryptophan is the only phosphorescing chromophore over this time range.

The largest differences in the decays, compared to the pseudo wild type HCA II, are seen in the W209F/C206S, W5F/W16F/C206S, W245C/C206S and W97C/C206S mutants where the

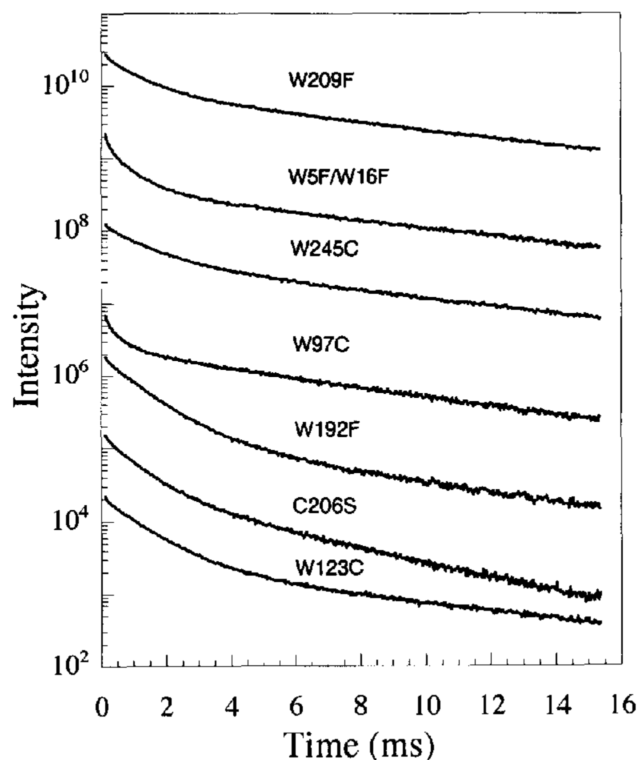

Fig. 1. Phosphorescence decays (20°C) of HCA II mutants observed at 440 nm following pulsed excitation at 280 nm. Each transient represents 1000 laser shots. The decays are offset by one log unit from each other for clarity, and hence the intensity units are completely arbitrary.

relative contributions of short-lived components to the total decay are decreased (Fig. 1). It is very surprising though, that the longest life-time components do not differ very much among the mutants. Indeed, the long life-time component of the pseudo-wild type C206S mutant is even noticeably diminished relative to the other mutants (Fig. 1). The phosphorescence intensities (Fig. 2) differ somewhat between the various mutants though we note that the phosphorescence from the W209F/C206S mutant is considerably enhanced compared to the rest. This could be due to singlet–singlet energy transfer via W209 to some non-phosphorescing residue, thus competing with intersystem crossing to populate the triplet-state; W209 has been suggested to be involved in energy transfer to H107 and the H119-Zn^{2+} complex resulting in quenching of the fluorescence of HCA II [5]. A singlet-singlet energy transfer would decrease the intensity due to reduced emission from W209 as well as due to quenching of the remaining Trps. However, singlet–singlet energy transfer would not be expected to affect the decay kinetics. The difference in the decay between W209F and C206S indicates the possibility that W209 partially quenches other phosphorescing residue(s), a process that is exponentially dependent on the donor–acceptor pair distance [19]. The closest distance between the aromatic rings of W209 and the other Trp residues are: W192, 9.7 Å; W5, 12.9 Å; W16, 12 Å; W97, 13.5 Å; W245, 12 Å; W123, 16 Å. Based solely on a proximity argument, triplet state quenching of W192 by W209 is possible, but since a similar increase in the long life-time component is evident irrespective of which Trp residue is removed, the change in decay kinetics could instead be due to

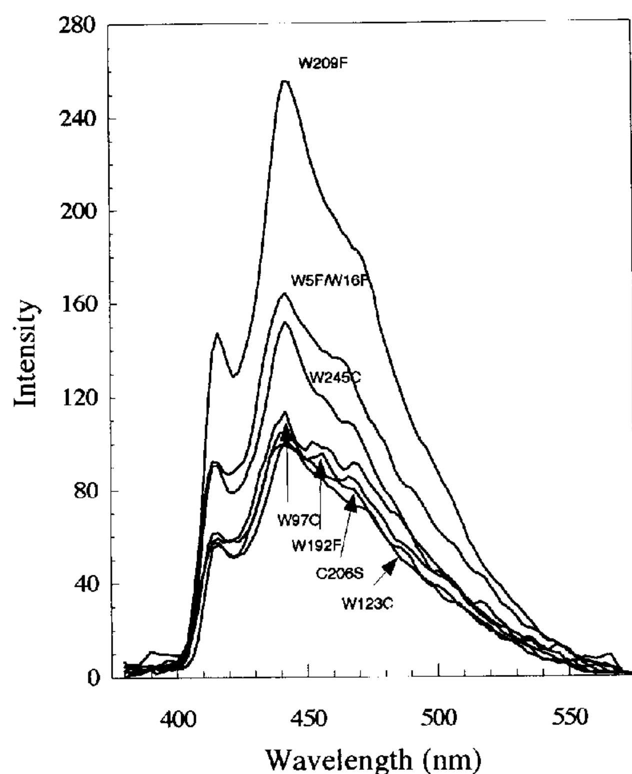

Fig. 2. Phosphorescence spectra (not corrected) of HCA II mutants at 4°C with 4.8 nm bandpass. The proteins were excited at 280 nm with a repetition rate of 10 Hz, and the emission intensities were observed for a gate of 99.9 milliseconds beginning 100 microseconds after excitation. The integrated intensities for equal concentrations of protein relative to the most intense W209F spectrum are 0.70, 0.58, 0.50, 0.46, 0.44 and 0.41 for W5F/W16F, W245C, W97C, W192F, C206S and W123C, respectively.

slight conformational changes in the mutants. The increase in phosphorescence intensity in the case of removal of W5 and W16 can possibly be explained by similar mechanisms as discussed for W209; W5 has been found to be involved in quenching of the fluorescence via energy transfer to H64.

The long-lived phosphorescence from many multi-tryptophan containing proteins is thought to arise from a single tryptophan residue in a buried, rigid environment [9]. In the present case, in spite of some differences in the observed intensities, it is evident that the emission cannot be assigned to a single tryptophan since there are only slight changes when each of all the Trp in the protein are sequentially removed. Evidently, there must be at least two phosphorescence emitting Trp, with similar life-times of around 9.3 ms.

One has to bear in mind that the very subtle differences seen in the phosphorescence decays and intensities might to an extent be due to slight conformational differences between the different mutants; minor conformational differences seem likely to occur, since the mutants have specific activities that vary from 40 to 100% [5], in spite of the fact that none of the mutated tryptophan residues are thought to be involved in the catalytic mechanism. Since the specific activities still are very high, the conformational differences must be minor, but like the enzymatic activity, the RTP is very sensitive to slight conformational changes; for this reason the assignment of the small differences in RTP to specific tryptophan residues is unwarranted.

In summary, the results presented here clearly show that although one tryptophan residue, W97, is deeply buried in a probably rigid environment, the removal of this residue does not change the long lived RTP decay component. The usefulness of this technique as a probe for protein structure and folding studies is dependent on the ability to correlate the RTP with specific residues, and it is evident that one has to be careful in assigning room temperature phosphorescence decay-components to residues based solely on crystallographic structure or on the selective deletion of possible chromophores.

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