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Electron transfer from excited tryptophan to cytochrome *c*: mechanism of phosphorescence quenching?

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Parvalbumin, aldolase and liver alcohol dehydrogenase (ADH), proteins exhibiting long-lived phosphorescence lifetimes at room temperature, were examined for their reactivity with ferricytochrome *c* (cytochrome *c* Fe^{3+}) as an external electron acceptor. Illumination of a reaction mixture containing protein and cytochrome *c* in the absence of oxygen brought about reduction of cytochrome *c* in relation to the duration of light. The largest portion of reduced cytochrome *c* was found with a sample containing ADH, where a 50% reduction of cytochrome *c* was reached after 5 min of illumination with a xenon lamp. Parvalbumin and aldolase were about half as effective under the same conditions. Several lines of evidence support the idea that the reaction of cytochrome *c* occurred by a long-range electron transfer from the excited triplet state of tryptophan. First, cytochrome *c* quenches the tryptophan phosphorescence and with parvalbumin, its bimolecular quenching rate constant, k_q, was $2.9 \cdot 10^6$ M^{-1} s^{-1}. Second, when the illuminated reaction mixture was supplied with 0.2 mM to 1 mM nitrite, a concentration range of nitrite which quenches the tryptophan phosphorescence but not the fluorescence, the amount of reduced cytochrome *c* on illumination markedly decreased. Finally, for all illuminated protein samples, the extent of cytochrome *c* reduction occurred parallel to a decrease in tryptophan content as judged from a decrease in fluorescence intensity and/or a decrease in tryptophan absorption at 280 nm.

Introduction

Most proteins absorb light at 280 nm due to the presence of the aromatic amino acid tryptophan which possesses an indole ring. The process of absorption transforms indole into an excited species, which is more reactive than the ground-state molecule [1]. Reactions of the singlet and triplet excited state species can be monitored by fluorescence and phosphorescence, respectively. While tryptophan fluorescence has been used to study protein structure for many years [2,3], tryptophan phosphorescence measurements at room temperature were recently made possible by working under exclusion of oxygen [4–6]. The phosphorescence of some proteins could be attributed to one or a very small number of tryptophan residues buried in the protein [7], making their identification and location possible. This allows a meaningful interpretation of data obtained by phosphorescence measurement and their applicability as a tool to study protein dynamics and conformation [8]; for review see Ref. 9.

Specific interactions between excited state molecules and neighboring molecules may be explained by dipolar (Forster) interactions and electron-exchange/transfer reactions. In recent studies [10] it was shown that tryptophan protected in the core of protein can interact with quencher by a reaction that occurs over distance. By examining the distance dependence of quenching, it was concluded that some non-colored quenchers probably interact by electron transfer/exchange reaction [11]. Mersol et al. [12] observed that the phosphorescence quenching rate of alkaline phosphatase by freely diffusible large molecules of dyes and hemoproteins was virtually independent of the overlap of the emission and absorption spectra. When used in reduced state, the dyes exhibited a much lower quenching although the overlap integral was not substantially changed. This was taken as evidence that long-range electron transfer dominates the quenching by quenchers in an oxidized state.

A general support for the occurrence of long-range electron transfer between proteins could be obtained

Abbreviations: ADH, liver alcohol dehydrogenase; Trp, tryptophan.

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by examining the electron transfer from several protein macromolecules exhibiting long-lived phosphorescence to a protein with a readily measureable redox center. Parvalbumin, aldolase and liver alcohol dehydrogenase (ADH) where the long-lived phosphorescence has already been assigned to a particular tryptophan residue [7,9], where chosen. They differ in phosphorescence lifetimes in the range of an order of magnitude and the location of the emitting tryptophan residue in the structured region of the protein has recently been estimated [13]. Hemoproteins possessing an embedded heme group seem to be suitable candidates for electron acceptors from the excited triplet state modeling native interactions among macromolecules [12,14]. Their reactions can mimic the electron transfer reactions which native heme proteins undergo.

Cytochrome *c*, a monomeric globular heme protein, is well suited to serve as a reactant to study the intermolecular transfer of electrons [12,15,16]. It has a single buried tryptophan that is adjacent to the heme. Singlet energy transfer from this tryptophan to the heme is nearly 100% effective; consequently, the yields of its fluorescence and phosphorescence are too small to interfere in the measurement. Cytochrome *c* has a relatively high redox potential and its reduced and oxidized forms can be easily distinguished. In the present paper, we show that cytochrome *c* can be used as an effective acceptor of an electron from a triplet excited state of indole in other proteins.

Materials and Methods

Materials

Alcohol dehydrogenase from equine liver (1.63 units/mg protein), aldolase (Type X) from rabbit muscle, cytochrome *c* from horse heart (Type VI, MW 12,384) and sodium nitrite LOT 123F were from Sigma. Parvalbumin fractions differing in tryptophan content were prepared from frozen cod fillets according to Ref. 17 with some modifications described previously [18].

Methods

1.4 ml of reaction mixture (composition given in the legends) in a spectrophotometric quartz cuvette containing 0.5–0.8 mg of protein was made anaerobic by adding solutions bubbled with oxygen-free argon [5] and using an enzyme deoxygenating system [11]. Into the reaction mixture 10 μl of anaerobic solution of 1 mM cytochrome *c* Fe^{3+} was injected. The samples were illuminated using unfocused Perkin-Elmer 150 W Xenon lamp at 10 cm distance with a liquid filter containing 5% $CuSO_4$; this filter transmits less than 1% light at 280 nm and below and about 3% at 290 nm and 10% at 295 nm, and it also eliminates infrared light. The optical pathlength for illumination was 0.2 cm. Before and after illumination absorption spectra in the range of 240 nm to 600 nm and fluorescence emission spectra in the range of 300–400 nm using 282 nm for excitation were registered. An excitation wavelength of 282 nm was used. The emission was measured between 300 and 400 nm.

To measure triplet state quenching (Fig. 2), phosphorescence emission was measured at 440 nm as described previously [14].

For all experiments the temperature was 22°C.

Instrumentation

Absorption spectra were measured with a Perkin-Elmer-Hitachi 200 spectrophotometer. Fluorescence spectra were obtained with a Perkin-Elmer 650-10 S fluorescence spectrophotometer and a Perkin-Elmer LS-5 luminescence spectrophotometer. Phosphorescence lifetimes were measured by using the instrument described by Vanderkooi et al. [6].

Results

The induction of cytochrome c reduction by tryptophan-containing proteins on illumination

Parvalbumin, aldolase and ADH, with phosphorescence lifetimes of 5, 45 and 300 ms, respectively, were chosen as representatives of proteins containing tryptophan with 'moderate' but different accessibility [9]. Illumination of each of the tryptophan containing proteins in a deoxygenated mixture containing oxidized cytochrome *c* brings about cytochrome *c* reduction. Typical records of the effects of individual proteins on the extent of cytochrome *c* reduction by illumination are given in Fig. 1, which shows the results of experiments where ADH, and two fractions of parvalbumin were used. After 5 min of illumination, cytochrome *c* becomes partly reduced and the extent of reduction further increases with a prolonged illumination. The largest effect was observed in the experiment with ADH (see Fig. 1, trace 4), where a 5 min illumination caused 36 to 53% reduction, the additional 15 min illumination resulted in 68 to 78% reduction of cytochrome *c* in the sample.

In the experiment with aldolase (not shown), the observed values of cytochrome *c* reduction were 16 to 24% and 32 to 43% after 5 min and 20 min of illumination, respectively. Fig. 1, traces 2 and 3, display experiments with two fractions of parvalbumin differing in the content of tryptophan [17]. The one fraction composed mostly of parvalbumin III (which contains one tryptophan residue) brought about 22% and 28 to 32% reduction; the other fraction, comprising mostly parvalbumin II (which is devoid of tryptophan), was much less effective and showed no clear dependence of the reduction of the length of illumination. Illumination of the tryptophan free parvalbumin caused only about 10% reduction of cytochrome *c* after 5 min and the extent did not increase after 20 min of illumination.

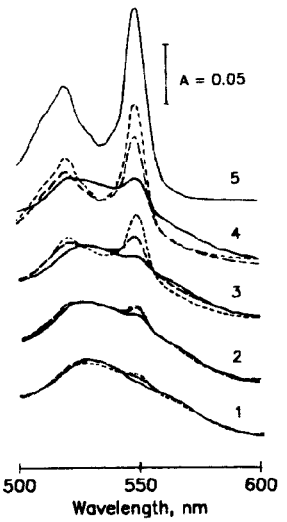

Fig. 1. Cytochrome *c* reduction in the presence of tryptophan containing proteins. Long-wavelength absorption spectra of samples of 6.9 μM cytochrome *c* Fe^{3+} and 0.5 mg to 0.8 mg of protein in 0.1 M NaCl and 0.01 M phosphate (pH 7.0) devoid of oxygen (for 1, 4) or in 0.1 M NaCl plus 0.01 M Tris-HCl buffer (pH 7.0) supplemented with 1 mM $CaCl_2$ (2, 3) on illumination: ——— 0 min; – – – 5 min; ------ 20 min. (1) No protein added; (2) parvalbumin II; (3) parvalbumin III; (4) liver alcohol dehydrogenase; (5) reduced with $S_2O_4^{2-}$. The sample also contained the oxygen-scavenging system: 80 nM glucose oxidase, 16 nM catalase and above 10 mM glucose.

In control experiments, samples of cytochrome *c* without protein addition were illuminated. We found that 5 to 7% of the given amount of cytochrome *c* Fe^{3+} may undergo autoreduction after 20 min of illumination (Fig. 1, trace 1). We also examined whether the proteins themselves induced reduction of cytochrome *c* in the absence of light during the deoxygenating procedure. From the traces exhibited in Fig. 1 it is seen that the portion of reduced cytochrome *c* before illumination in the presence of protein differed slightly in individual samples. The effect was most pronounced with ADH (Fig. 1, trace 4) where as much as 19% of the cytochrome *c* Fe^{3+} in the mixture may be in the reduced form at the begining of the experiment. We have not identified the reducing agent present in the samples with ADH, and in evaluating the effect of light, we only considered the additional effect produced by illumination.

Cytochrome c quenches the tryptophan phosphorescence of parvalbumin

The effect of cytochrome *c* on the phosphorescence of parvalbumin is depicted in Fig. 2. From the decrease in its lifetime caused by an interacting molecular species, the quenching rate constant, k_q, can be calculated using the Stern and Volmer equation modified for estimating lifetimes [11]. Cytochrome *c* in a 33 μm concentration quenches the phosphorescence of parvalbumin approx. 25% and produces a bimolecular quenching rate constant of $3 \cdot 10^9$ M^{-1} s^{-1}.

The reduction of cytochrome c is diminished by a substance that quenches phosphorescence

On the assumption that the electron transferred from protein to cytochrome *c* originates from the excited triplet state of tryptophan, the presence of a quencher of the triplet state should decrease the reaction. Nitrite was selected for the experiment as a low-molecular-weight substance free to difuse in solution but, as a charged molecule, it would not penetrate the protein interior [11]. Nitrite itself quenched tryptophan fluorescence with quenching constants (k_q) of $2 \cdot 10^9$ M^{-1} s^{-1} and $4 \cdot 10^9$ M^{-1} s^{-1} for ADH and parvalbumin, respectively [18]; however, because the singlet lifetime is only about 5 ns the fluorescence intensity of the two proteins decreased by less than 1% at the concentration most frequently used, 0.2 mM nitrite. The effect of the presence of nitrite in the reaction mixture of protein and cytochrome *c* at illumination is shown in Fig. 3. The figure shows the absorption spectra of samples with ADH and parvalbumin illuminated for the same period and measured as a difference between a sample where protein and cytochrome *c* were supplemented with nitrite and an original mixture containing only protein and cytochrome *c*. It can be seen that the presence of nitrite in the illuminated sample results in decreasing the cytochrome *c* reduction. A similar effect of nitrite presence was also found in a sample with aldolase (not shown). The effect of nitrite was most apparent for the proteins that gave the largest reduction of cytochrome *c* upon illumination. The amount of reduced cytochrome *c* in samples containing nitrite was about

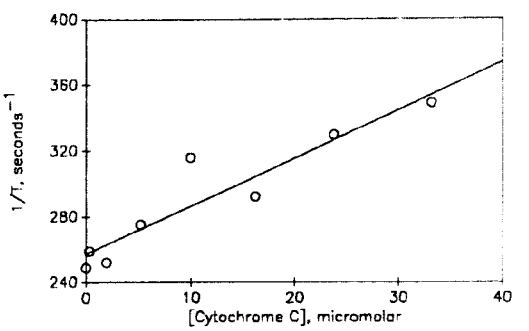

Fig. 2. Stern-Volmer plot for quenching of parvalbumin phosphorescence by cytochrome *c* Fe^{3+}. A sample of parvalbumin III containing 0.7 mg protein in 1.4 ml of deoxygenated solution of 0.1 M NaCl, 0.01 M Tris-HCl (pH 7.0) and 1 mM $CaCl_2$ was titrated with 1 μl to 30 μl additions of 0.5 mM cytochrome *c* Fe^{3+} and phosphorescence lifetimes were measured. Data are plotted according to Re.. 10 to obtain the second-order quenching rate constant, k_q, from the slope.

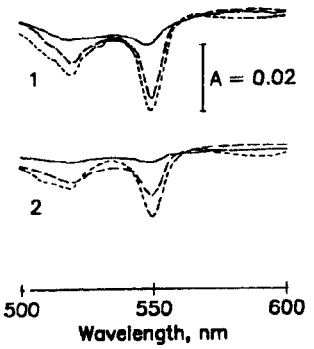

Fig. 3. Protective effect of nitrite on the cytochrome *c* reduction. Absorption spectra of reaction mixture (see Fig. 1) supplemented with 0.2 mM nitrite (sample cuvette) were measured as difference against an original sample (reference cuvette). (1) Liver alcohol dehydrogenase; (2) parvalbumin III. The intervals of illumination and the symbol designations were as for Fig. 1.

80% of the amount of a sample run in parallel without nitrite (values as low as 71% were also observed). More experiments will be needed to obtain a quantitative evaluation of the nitrite effect with individual protein, which was not the aim of the present study. The evaluation of the nitrite effect with time has shown that with proteins tested, except the parvalbumin II fraction, the protective effect of nitrite is maintained during the course of illumination. Some differences, however, may exist in the kinetics of protection as indicated by the different proportion of curves of absorption obtained after 5 min and 20 min of illumination with ADH and parvalbumin (Fig. 3). In the sample containing the tryptophan poor fraction of parvalbumin, the effect of nitrite could hardly be observed and was detected only after an extended period of illumination.

As an oxygen-containing molecule with an N^{3+} atom, nitrite is a reactive chemical agent which has an ability to react with some hemoproteins. To exclude the possibility that the decrease in cytochrome *c* reduction on illumination is due to direct interaction between nitrite or some of its reaction products with cytochrome *c*, control experiments were undertaken where nitrite and cytochrome *c* were incubated under conditions of experiment. No change in absorption spectra of cytochrome *c* was observed. Conversely, when a sample with nitrite and cytochrome *c* was illuminated for 5 min and 20 min, 7 to 16% and 13 to 23%, respectively, of cytochrome *c* was found in the reduced state (not shown). This means that nitrite alone is also partially effective in inducing the cytochrome *c* reduction. It is obvious that the lower extent of cytochrome *c* reduction observed in the sample containing both nitrite and protein cannot be ascribed to interaction of nitrite with cytochrome *c* and may be attributed to its quenching capability.

Fluorescence emission of tryptophan residues on illumination

The illumination of protein in a mixture with cytochrome *c* results in a decrease in the Trp fluorescence emission. The change was observed with all proteins which were effective in inducing the cytochrome *c* reduction. The decrease of fluorescence emission measured after the two intervals of illumination was related to the extent of cytochrome *c* reduction in the sample. The largest decrease of fluorescence was observed with ADH (Fig. 4, A) and smaller decreases in the case of aldolase and parvalbumin were seen (not shown). For parvalbumin II no change in emission intensity was observed at all. As shown in a more precise measurement with renormalized intensity of emission (Fig. 4, B), the illumination of ADH with cytochrome *c* causes a gradual decrease of a fluorescence emission at shorter wavelengths (around 330 nm) and a red shift of 3 nm of the maximum. The illumination of ADH alone does not show a measurable change in fluorescence emission. The decrease in tryptophan fluorescence of all proteins effective in cytochrome *c* reduction in the course of illumination supports the view of an irreversible change in tryptophan residues, apparently due to an electron transfer reaction to cytochrome *c*.

Change of absorption in the ultraviolet region

Tryptophan absorption at 280 nm presents another possibility to evaluate its participation in the observed electron transfer reaction. In our experiments the illumination of protein samples lacking cytochrome *c* has proved to have practically no effect on absorption in

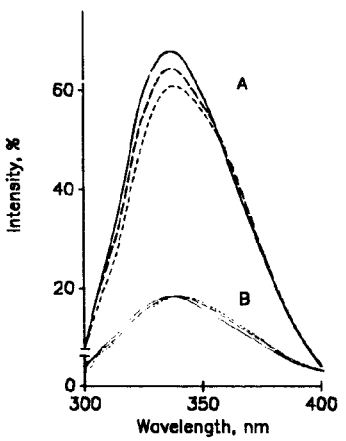

Fig. 4. Fluorescence emission spectra of ADH illuminated with cytochrome *c*. (A, B) Emission spectra after 0 min, 5 min and 20 min of illumination with renormalized intensity respectively. The composition of samples and meanings of symbols were as described in Fig. 1; the excitation wavelength was 282 nm. The measurements were performed as indicated in Materials and Methods.

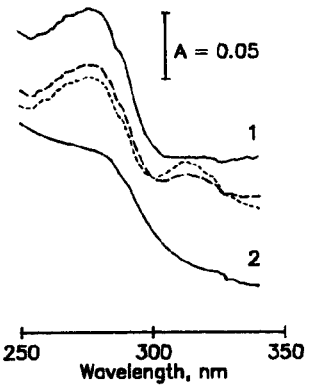

Fig. 5. Changes in absorption in the ultraviolet region on illumination of ADH and cytochrome *c* Fe^{3+}. Absorption of a sample of 4.6 mM cytochrome *c* Fe^{3+} and 0.6 mg of ADH illuminated as stated in Fig. 1, (1); and a sample after 20 min illumination and reoxidation of cytochrome *c* by ammonium persulfate (2). The oxidation was complete as indicated by the absorption spectrum. Symbol designations are as in Fig. 1.

the ultraviolet region (not shown). According to the results given above it could be expected that in illuminated samples, a reduction of cytochrome *c* will be accompanied by a decrease in tryptophan absorption. This was difficult to prove because of the interference of emerging delta band absorption of cytochrome *c* Fe^{2+} and a known additional absorption of cytochrome *c* Fe^{2+} at 270 nm [19]. The expected drop in the 280 nm absorption due to the Trp damage is countered by the increase in absorption of these two bands as demonstrated in Fig. 5, trace 1. The absorption phenomena in this region are complex and do not permit a quantitative evaluation of the amount of tryptophan irreversibly changed owing to the redox reaction with cytochrome *c*. Nevertheless, by reoxidizing the reduced cytochrome *c* in the illuminated sample, ADH showed evidence of a decrease in tryptophan absorption at 280 nm during the cytochrome *c* reduction (Fig. 5, trace 2).

Discussion

The triplet state from which tryptophan phosphorescence originates is formed by intersystem crossing from the first excited singlet state. It is now commonly accepted that long-lived tryptophan phosphorescence is related to local structural rigidity [7] and is exhibited by tryptophans located in highly structured regions of protein [9,11]. In most proteins only a small number of tryptophans, or even one, fulfill the requirement and phosphoresce significantly. The identification of the emitting tryptophans in some proteins has already been achieved and their distance from protein surface estimated [12,13].

The phosphorescence lifetime depends upon non-radiative processes, including vibrational losses, transfer to internal moities such as sulfur, metal or colored centers. In addition, a quenching term arises from the interactions with quencher species bound to the protein or free to diffuse in the reaction medium. Up to now, most of the studies were devoted to exploring the mechanism of quenching brought about by external small molecules. The results obtained with 'large' (i.e., > 3 atoms) quenchers led the authors [11] to consider long-range transfer reactions which can occur either through a dipolar [20] or through an electron-exchange/transfer mechanism [21]. Cytochrome *c*, with one of its heme edges exposed to the protein surface, yields the closest approach distance which lies in the range for exchange interactions. Its absorption spectrum partly overlaps with the long-lived emission of tryptophan of the interacting protein. The overlap integral of the reduced heme is only slightly (about 5%) larger than that of its oxidized species [12] which means that it can be used as a probe in resonance energy transfer measurements as well as for examining long-range electron transfer/exchange reactions.

In our experiment, cytochrome *c* was found to quench the parvalbumin phosphorescence (see Fig. 2). The second order quenching constant k_q was $2.9 \cdot 10^6$ M^{-1} s^{-1}. In a recent work of another laboratory [12] it was found that cytochrome *c* quenches the phosphorescence of alkaline phosphatase, whose tryptophan is deeply buried, with an overall quenching of $2.5 \cdot 10^2$ M^{-1} s^{-1}. Parvalbumin is also much more susceptible to phosphorescence quenching than alkaline phosphatase by a variety of small molecules, which is consistent with the known location of tryptophan in parvalbumin near the protein surface [10]. Mersol et al. [12] found that the oxidized form of cytochrome *c* was more effective in quenching than the reduced form which was also taken as an evidence that a long-range electron transfer is the main quenching mechanism.

A question arises whether the buried tryptophan is responsible for the observed electron transfer. In the case of parvalbumin, there is a single Trp moiety, but aldolase has three Trp per subunit and ADH has two per subunit. It is possible that excited state reactions occur from the short-lived (and not detected by us) excited triplet state of the other Trp moieties in the protein. It was found that the observed reduction increased in the sequence of parvalbumin < aldolase < ADH, which is the same sequence for phosphorescence lifetime and burial, suggesting, but not proving, that the buried Trp is responsible. A further clue to this is found in an interesting feature in the reaction of ADH, witnessed by the change in the fluorescence emission of the illuminated sample (see Fig. 4, B). A red-shift of the maximum of the fluorescence emission was observed in the total emission. In terms of tryptophan classification [3] this would mean that the mi-

croenvironment of the emitting tryptophan residue is more hydrophilic, and is consistent with the idea that the single buried tryptophan of ADH is destroyed preferentially over the single exposed Trp. The buried tryptophan therefore appears more reactive than the exposed tryptophan. Since the lifetime of the exposed Trp is short because of competing quenching mechanisms, and for kinetic reasons it may be not able to transfer electrons to cytochrome *c* in solution. We would like to put forward another possibility. If the quenching reaction occurs basically by an exchange/transfer mechanism, the reverse reaction can occur in the excited state. In this scheme, there would be quenching of phosphorescence, but no net electron transfer. Intuitively, this can be seen to be possible, since when the donor and acceptor are close, both electron transfer to and from Trp would be fast. In the case of the buried Trp, transfer rates are slow. This means that the reaction is in the rapid diffusion limit where the excited state molecule senses the average concentration of cytochrome *c*, but not the diffusion of cytochrome *c* [22]. The reverse reaction would also be slow, but in this case, the reduced cytochrome *c* has time to diffuse away and, since it is not replaced by a reduced cytochrome *c* molecule, a net reaction will be observed. We need to point out that when the previously illuminated sample was allowed to stand in the dark, we did not observe reoxidation of the reduced cytochrome *c*. The reoxidation in the ground state also may not occur because of thermodynamic reasons – the redox potentials may be unfavorable, and identification and characterization of the indole product would be necessary to prove this. There may also be a kinetic barrier. The reaction between an excited triplet state Trp and paramagnetic cytochrome *c* Fe^{3+} is spin allowed, whereas the ground state reaction between the presumed triplet radical and cytochrome *c* Fe^{2+} is spin-forbidden.

Further support for participation of tryptophan triplet state in cytochrome *c* reduction comes from the ability of nitrite to slow down the effect. In Fig. 3, traces 1, 2, we show that presence of nitrite in a reaction mixture with ADH and parvalbumin results in a marked decrease in cytochrome *c* reduction. The protective effect of nitrite appears to be related to phosphorescence quenching [11,13]. The phosphorescence quenching constants, k_q, found for nitrite are in the range of 10^6 M^{-1} s^{-1} for parvalbumin and 10^4 M^{-1} s^{-1} for ADH and aldolase. It seemed to us to be well suited for determining if a quenching agent affects the reduction of cytochrome *c* during the illumination of the reaction mixture. Since in our experiments the fluorescence was not significantly quenched by the nitrite at the concentrations used, the decrease by about 20% in the cytochrome *c* reduction observed with proteins tested above is related to the quenching of phosphorescence, indicating that when the population of the triplet state is reduced, the electron transfer is also reduced.

A decrease in fluorescence emission was observed after illumination with all proteins which in excited state were effective as electron donors for cytochrome *c* reduction. The extent of change roughly was related to the extent of cytochrome *c* reduction and brings additional evidence for a transformation of the buried tryptophan residue caused by the loss of electron. This was further confirmed by following the light absorption in the UV region. As demonstrated with ADH in Fig. 5, traces 1, 2, a decrease in the absorption maximum at 280 nm was detected in the illuminated sample. Although the absorption changes in the UV region are complex owing to the appearance of cytochrome *c* Fe^{2+} absorption in the delta region [19], the reoxidized sample clearly shows the decrease in tryptophan content. The result is in accordance with our assumption that long-range electron transfer from tryptophan triplet state is functional in cytochrome *c* reduction.

Acknowledgements

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