THE REACTION OF PSEUDOMONAS AZURIN WITH HYDRATED ELECTRONS

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SUMMARY

The reaction of azurin, the bacterial "blue" copper protein, with hydrated electrons has been investigated by the pulse radiolysis technique. The single Cu(II) ion of this protein was found to be reduced in a direct bimolecular reaction with the hydrated electron at a specific rate of $1.0 \pm 0.3 \times 10^{11} M^{-1}sec^{-1}$. The observed reduction rate is very close to the theoretical diffusion controlled limit. The observed direct reduction mechanism is different from that observed for Cu(II) reduction in "blue" copper oxidases. The yield of specific reduction of the Cu(II) site of azurin is small as a result of competing side reactions with amino acid residues of the protein.

INTRODUCTION

The azurins form a class of proteins of relatively low molecular weight (16000 daltons). They are abundant in certain bacteria of the genera: Pseudomonas, Bordetela and Alcaligenes (1-3). The single copper ion of these proteins is characterized by its strong absorption band centered at 625 nm and the exceptionally narrow hyperfine splitting of its electron spin resonance spectrum (4-6). This type of Cu(II) ions was first characterized in the copper oxidases laccase and ceruloplasmin which have three different copper binding sites and was designated type 1 (1). Azurins are most probably electron mediating proteins involved in the bacterial cytochrome chain (7). Recent kinetic studies have shown its ability to undergo rather fast electron exchange with Pseudomonas cytochrome c 551 (8). In the present study the reaction of the hydrated electron (e^-_{aq}) with Pseudomonas azurin has been undertaken using the pulse radiolysis technique. This technique makes it possible to produce either oxidising (OH free radicals) or reducing (e^-_{aq}) species in the bulk of the examined solution via the interaction of energetic electrons with water, within the time range of less than 1 μsecond (9, 10). Recently this technique was applied to the study of electron transfer mechanism of two different redox proteins (11, 12),

namely, horse heart cytochrome-c and fungal laccase. All "blue" copper oxidases contain several copper ions bound to one protein molecule, one of them at least of the type 1. Azurins containing only a single copper ion forms a convenient system for the study of the electron transfer process to the type 1 Cu(II).

MATERIALS AND METHODS

Azurin was prepared from Pseudomonas fluorescence according to the method described by Ambler (13, 14). The purity of the preparation was checked by starch gel electrophoresis (15). Tertiary butanol was analytical grade (Merck AG, Darmstadt). All other reagents used were at least analytical grade purity. Solutions were freshly prepared before each experiment by diluting the stock protein solution in triple distilled water from which oxygen was previously removed by continuous bubbling of highly purified argon. All solutions also contained 0.1 M tertiary butanol which reacted with all OH radicals and converted them into the relatively unreactive $CH_2C(CH_3)_2OH$ radicals. Gas chromatographic analysis of the dissolved gas showed $[O_2] \leq 10^{-7}$ M. No buffer was used and the pH was adjusted to 7.0 by the addition of acid or base.

The pulse radiolysis system of the Hebrew University of Jerusalem was used (20). A linear accelerator operated at 200 mA and 5 Mev served as the electrons source. The concentration of the produced hydrated electrons was varied by changing the pulse length. In this study pulses of 0.1 to 1 μsec length were used. By ferrocyanide dosimetry (16) these pulses were found to produce 0.65 to 5.1×10^{-6} equivalents/lit of e^-_{aq}.

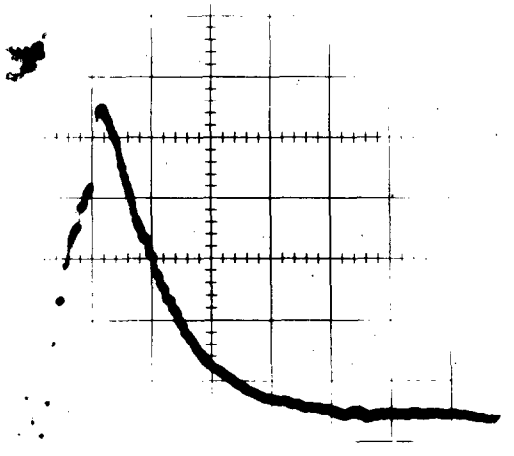

Fig. 1: Osciloscope trace of the decay of e^-_{aq} followed at 550 nm in azurin = 1.25×10^{-5} M. Sweep rate was 1 μsecond/scale unit and sensitivity = 50 mV/scale unit. $e^-_{aq} = 6.5 \times 10^{-7}$ equivalents/lit

RESULTS AND DISCUSSION

An osciloscope trace of the decay of the hydrated electron absorption observed at 550 nm in argon saturated 6.2×10^{-6} M azurin is presented in Fig. 1. This process is pseudo first order and the specific rate constant was calculated to be $1.0 \pm 0.1 \times 10^{11}$ $M^{-1}sec^{-1}$. The theoretical limit for a diffusion controlled reaction may be calculated using the Debye equation (17):

$$k_{diff} = \frac{4\pi N}{1000} (D_e + D_p)(r_e + r_p) Q/(e^Q - 1)$$

where $Q = Z_e \cdot Z_p - e^2/\epsilon kT(r_e + r_p)$;

$\epsilon = 78.6$, the macroscopic dielectric constant of water at 25°C;

N = Avogadro's number 6.025×10^{23} molecules/mole;

k = Bolzman's constant, 1.38×10^{-16} erg/degree;

D_e and D_p are the diffusion coefficients of e^-_{aq} and the protein, respectively;

$D_e = 4.7 \times 10^{-5}$ $cm^2 \cdot sec^{-1}$. $D_p = 0.14 \times 10^{-5}$ $cm^2 sec^{-1}$.

We used 2.5 Å and 17 Å as the radius of e^-_{aq} (r_e) and the protein (r_p), respectively. $Z_e = -1$ is the charge of the hydrated electron. An average single positive charge was also assumed for the protein Z_p (14). The calculated value of $k_{diff} = 7.9 \times 10^{10}$. $M^{-1}sec^{-1}$ is in reasonable agreement with that observed experimentally.

At 625 nm where both the produced e^-_{aq} (18) and the type 1 Cu(II) absorb light, the transmittance changes also follow a pseudo first order curve. The dominant upper part of the curve is due to the decay of e^-_{aq} absorption ($\varepsilon_{625} = 1.2 \times 10^4$ $M^{-1}cm^{-1}$)

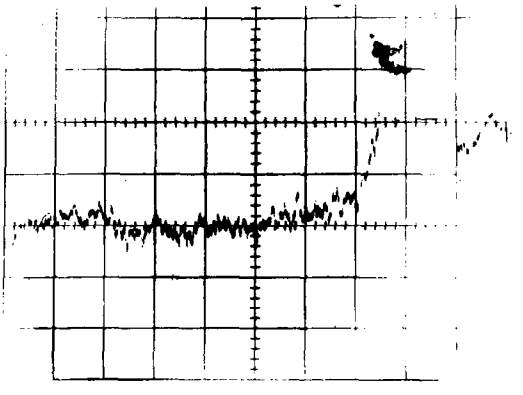

Fig. 2: Osciloscope trace of the type 1 Cu(II) reduction observed at 625 nm. The upper part which represents the decay of e^-_{aq} is not shown. Azurin = 3.6×10^{-6} M. Sweep rate is 5 μseconds/scale unit. Sensitivity = 5 mV/scale unit. $e^-_{aq} = 6.5 \times 10^{-7}$. equivalents/lit

and follows the same rate as that at 550 nm. The reduction of the Cu(II) ion is expressed in the lower part of the curve, namely, in the part decreasing below the prepulse absorption value (Fig. 2). Inspection of these decay curves and their computed kinetic analysis leads to the conclusion that the reduction rate of the Cu(II) is hardly distinguishable from that of the disappearance of e^-_{aq}. The low amplitude of the lower part of the decay curves (superimposed on the larger change due to e^-_{aq} absorption) causes a larger error ($\pm 30\%$) in the determined specific rate of Cu(II) reduction. Table 1 shows the experimentally obtained specific rate constants at different protein concentrations and at $[e^-_{aq}] = 1.2 \times 10^{-6}$ equivalents/lit. Clearly, the observed rates are very similar for the reduction of Cu(II) and e^-_{aq} decay.

Table 1: Bimolecular Rate Constants of the Decay of e^-_{aq} and Reduction of Type 1 Cu(II)

Azurin M	e^-_{aq} decay rate $M^{-1}sec^{-1} \times 10^{-11}$	Cu(II) reduction rate $M^{-1}sec^{-1} \times 10^{-11}$
5.7×10^{-6}	1.2 ± 0.1	1.0 ± 0.3
1.2×10^{-5}	1.1 ± 0.1	1.0 ± 0.3

All experiments done at neutral pH with no added salt. Tertiary butanol was 0.1 M. Temperature = $20 \pm 2^{\circ}C$.

During the electron pulse a transient absorption was found to appear in the near ultra violet region ($\lambda_{max} = 410$ nm). Its maximum and shape were very similar to the transient absorptions observed during the reaction of several proteins with the hydrated electron (19). This transient has been shown to be an $RS\dot{S}R^-$ radical ion formed by an electron attachment to a disulfide bridge. From our knowledge of the amino acid sequence of azurin (14) we are able to assign the absorbing transient to the reaction of the single disulfide bridge present in the positions 3-26 of the protein. This radical ion decays in a first order reaction (unaffected by protein conentration). The specific rate of this decay (Fig. 4) was found to be $1.4 \pm 0.2 \times 10^3$ sec^{-1}. This rate is of the same order of magnitude as that of the small slow decrease in the 625 absorption band which follows the initial fast reduction phase (Fig. 3).

A large proportion of the reducing equivalents are not utilized for the specific reduction of the Cu(II) ion. Under our conditions, the specific reduction yield amounts to only 20% of the produced hydrated electrons. It is mainly due to the direct reduction step and the contribution from the slow phase is rather small (Fig. 3). The loss of reducing

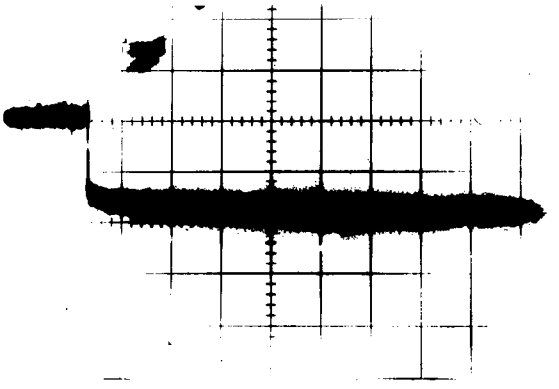

Fig. 3: Osciloscope trace of a slower scan of the Cu(II) reduction followed at 625 nm. Sweep rate is 1 msecond/scale unit. Sensitivity is the same as in Fig. 2. $e^-_{aq} = 6.5 \times 10^{-7}$. equivalents/lit

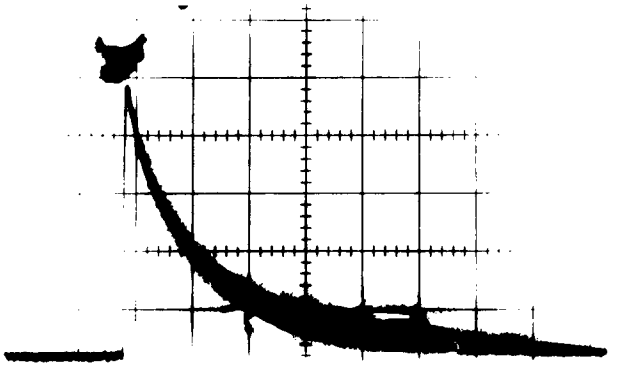

Fig. 4: Osciloscope trace of the decay of the absorption transient at 410 nm. Sweep rate is 500 μseconds/scale unit. Sensitivity = 10 mV/scale unit. $e^-_{aq} = 6.5 \times 10^{-7}$. equivalents/lit

equivalents on non-specific reaction with groups on the surface of the protein is a feature which has already been observed with other systems (20). It is a reflection of the exceptional reactivity of the hydrated electrons and probably also of the cross-section for the direct specific reduction.

The observed direct reduction of type 1 Cu(II) in azurin is the first example of such a mechanism. Laccase and ceruloplasmin were found to be reduced at their type 1 Cu(II) site with rates slower by more than two orders of magnitude. In these two cases the reduction proceeds via an indirect mechanism (12, 20): the hydrated electrons decay at a rate close to the diffusion controlled limit, either by reacting

non-specifically with exposed and reactive amino acid residues of the protein, or in a specific reaction with the electron accepting site of the oxidase. An intramolecular electron migration then brings about the reduction of the type 1 Cu(II). Also, some reduction takes place by an intermolecular electron transfer, i.e., from the initial site of its attachment to the electron accepting site of a second oxidase molecule.

Ferri cytochrome c was found to be reduced by the e^-_{aq} in a fast direct reaction (11) similar to that reported here for azurin. The direct reduction of cytochrome c is understood in terms of the availability of the heme group partly exposed in the open crevice of the protein. In analogy to the heme binding site in cytochrome c we may assume the existence of some crevice,or at least a channel in the structure of azurin, enabling the observed electron transfer to the Cu(II) ions. The relative ease of release of the copper ion from its binding site in azurin (21) is in contradistinction with the behaviour of the type 1 Cu(II) in laccase. Thus, the different availabilities of the copper ion in the two types of copper proteins are probably a main cause for their different reduction mechanisms.

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