OXIDATION–REDUCTION POTENTIAL OF UMECYANIN

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1. Introduction

Most copper-containing proteins are involved in oxidation–reduction systems, either as electron-transferring proteins or as oxidases [1, 2]. The copper ion(s) have been shown to be essential for the functions of all copper proteins studied.

Some recently isolated copper proteins belong to a family characterized by a molecular weight around 15,000 daltons, 1 Cu atom per molecule, and a very intense blue colour (Pseudomonas blue protein [3], azurin [4], stellacyanin [5], mung bean blue protein [6], rice bran blue protein [7]). Umecyanin, the most recently discovered member of this group, is isolated from horseradish root (*Armoracia laphatifolia*, Gilib) [8, 9] and has a molecular weight of 14,600 daltons [10]. Its spectral properties, as studied by CD and light absorption spectra [11, 12], indicate that the Cu atom is bound to the protein in a way very similar to that in Pseudomonas blue protein and azurin.

The functions of these proteins are so far unknown, but it is striking that the redox potentials are confined to a narrow range: +328 [1] and +275 [13] mV for the Pseudomonas and rice bran blue proteins, +395 mV for azurin [4], and $<+300$ mV for stellacyanin [14], all at pH 6–7. The purpose of the present investigation was to determine the redox potential of umecyanin.

2. Materials and methods

The umecyanin preparation [8, 9] was homogeneous in polyacrylamide gel electrophoresis and gave the ratio $A_{610}/A_{280} = 0.27$. Umecyanin, in the oxidized form after isolation, was dialyzed extensively against quartz-distilled water and lyophilized. The reduction of umecyanin was brought about by a slight excess of ascorbic acid, the resulting colourless solution being dialyzed extensively against quartz-distilled water and lyophilized.

Ascorbic acid (Merck, AG) was used without further purification. Samples were dissolved in oxygen-free water immediately before use. $Na_2S_2O_4$ and recrystallized $K_3Fe(CN)_6$ (Merck, AG) were dissolved in 30 mM sodium acetate, pH 5.85, which had been freed from air by means of argon bubbling for 2 hr. All solutions were stored in the dark under argon.

Spectrophotometric determinations were made with a Unicam SP 1800 spectrophotometer. The concentrations of reduced and oxidized umecyanin were calculated from the absorbancies at 280 and 610 nm, ϵ_{mM} being 12.7 and 3.4, respectively.

The oxidation–reduction potential was determined potentiometrically. An argon-filled, water-jacketted reaction vessel (25°) was used which permitted simultaneous spectrophotometry and potentiometry [15]. The potential between a bright platinum electrode and a saturated KCl–calomel electrode connected to the solution by an agar bridge with saturated KCl was measured by a Radiometer PHM 4 instrument. The calomel electrode potential was calibrated against an H_2-electrode and found to be +248 mV. Dichlorophenol indophenol (1% of the umecyanin concentration) was used as electron mediator; the contribution of its fully oxidized form to the light absorption at 610 nm was negligible.

3. Results

The dithionite solution was standardized against the ferricyanide solution by titration under spectrophotometric control (415 nm), exposure to light being minimized. Fig. 1 shows that 0.92 reducing equivalents per molecule of umecyanin were needed for complete reduction of the copper atom. The addition of ferricyanide in excess of previously added dithionite (fig. 1) caused a return of A_{610} to only about 0.8 indicating a partial destruction of the umecyanin by the excess of dithionite used to ensure full reduction.

To test the reversibility of the reduction, umecyanin was reduced with the milder reagent, ascorbic acid, and reoxidized by ferricyanide (fig. 2). With ascorbic acid the reduction was perfectly reversible and the reoxidation by ferricyanide proceeded rapidly. Umecyanin and ascorbic acid seem to react in the molar ratio 1:1.

The oxidation–reduction potential was determined with reduced and oxidized umecyanin dissolved in 20 mM sodium cacodylate, pH 7.0. Spectrophotometric determinations of the ratio oxidized/reduced umecyanin gave the value 0.26. Complete oxidation of

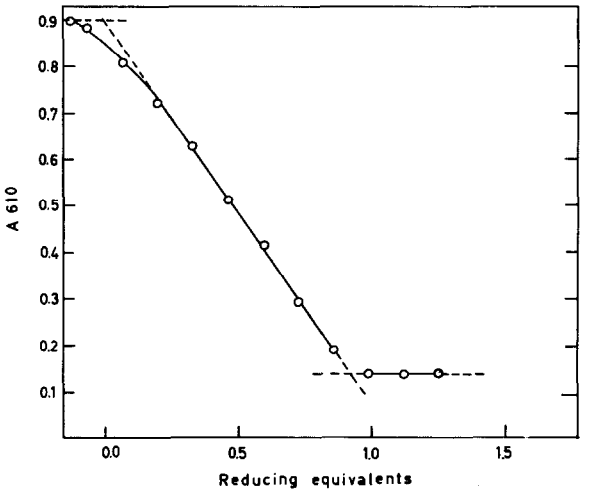

Fig. 1. Reduction of 2.5 ml 0.264 mM Cu^{2+}-umecyanin in 30 mM sodium acetate, pH 5.85, by the stepwise addition of 25 µl dithionite solution.

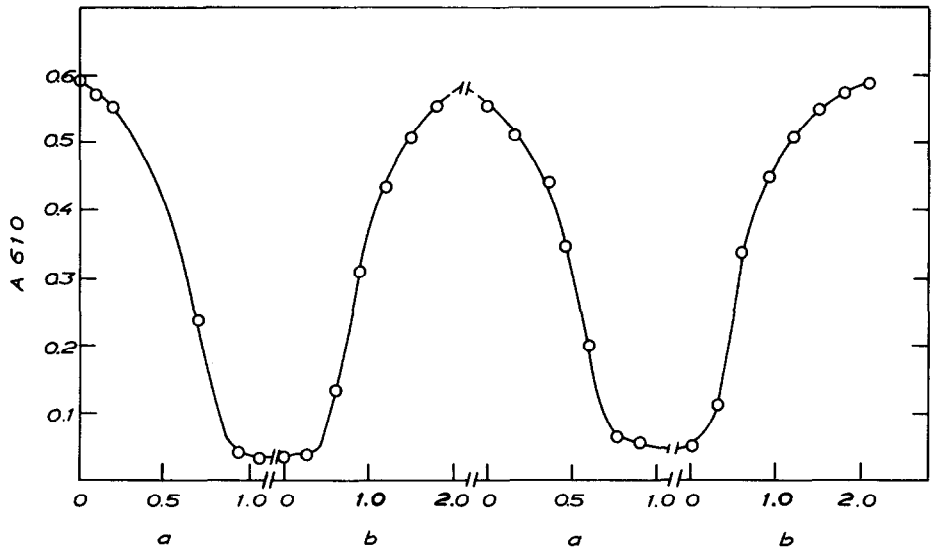

Fig. 2. Reduction and reoxidation of 1.0 ml 0.177 mM umecyanin in 0.1 M sodium cacodylate, pH 6.0, by stepwise addition of 8.6 mM ascorbic acid in the same buffer. 20 µl were consumed for complete reduction. The reoxidation was performed by the stepwise addition of 17.7 mM potassium ferricyanide. a) Moles of ascorbic acid/mole of umecyanin. b) Moles of ferricyanide/mole of umecyanin. 20 µl were needed for complete oxidation.

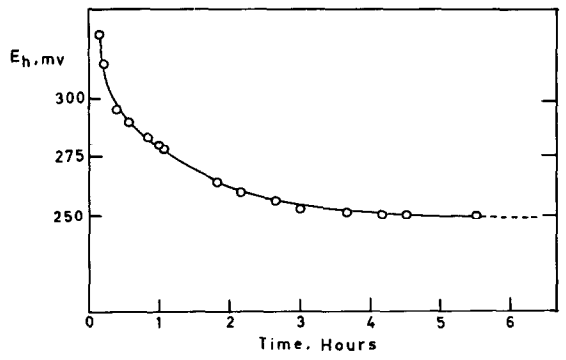

Fig. 3. Determination of the redox potential of umecyanin by direct potentiometry. The vessel contained 20 ml 0.59 mM umecyanin in 20 mM Na-cacodylate. Uc_{ox}/U_{red} = 0.26. pH = 7.0.

all umecyanin by means of ferricyanide after the experiment confirmed this ratio. Stable potentials were reached in about 6 hr (fig. 3). The half-oxidation potential was calculated from the usual formula

$$E_h = E'_0 + \frac{RT}{nF} \ln \frac{(ox)}{(red)}$$

and found to be +283.1 mV at pH 7.0, assuming n=1.

4. Discussion

The copper chromophore(s) responsible for the absorption maximum around 600 nm accept electron(s) in all the intensely blue proteins previously studied. The present investigation shows that umecyanin accepts only one electron in the copper chromophore (Cu^{2+}_{610}). The inability of the reduced umecyanin to be oxidized by molecular oxygen makes an oxidase function for umecyanin less probable. This is further supported by the spectrum of umecyanin, which lacks the absorption maximum around 330 nm found in all oxidase copper proteins so far. The reversibility of the oxidation–reduction reaction suggests that umecyanin is involved in electron transfer. The value E'_0 = +283 mV at pH 7, although one of the lowest recorded for an intensely blue copper protein, falls within the range found for the others. It is only slightly higher than that reported for cytochrome c (+250 mV at pH 6.8) [16], and there are, in fact, several similarities between the two chromoproteins: the electron-transfer, the redox potential, the molecular weight, and the existence of one atom of a transition element per molecule.

The observation that ascorbic acid is a one-equivalent reducing agent towards umecyanin under the conditions described in the legend to fig. 2 is startling and should be borne in mind when ascorbic acid is being used for reduction of copper proteins.

Acknowledgements

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