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Copper(I)-Incorporation into Spinach Apoplastocyanin

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Spinach plastocyanin was converted into the apoprotein. $CuSO_4$ and oxidized Cu(II)–thionein reacted with the apoprotein to Cu(II) plastocyanin. Cu(I) transfer from Cu(I)–thionein was only 15%. The structural analogue of the copper thiolate chromophore $[Cu(I)(thiourea)_3]Cl$ as well as $[Cu(CH_3CN)_4]ClO_4$ successfully formed the Cu(I)–holoprotein. Characteristic circular dichroism bands at θ_{284} (-5300 deg·cm^2·dmol^{-1}) and θ_{310} ($+3300$ deg·cm^2·dmol^{-1}) were seen. Upon oxidation with ferricyanide and dialysis against phosphate buffer the correct Cu(II) binding into the active centre of Cu(II) plastocyanin was confirmed by EPR-measurements. The use of $[Cu(I)(thiourea)_3]Cl$ as a convenient Cu(I) source for reconstitution studies on copper proteins is highly recommended.

Introduction

The molecular basis of copper transportation into plastocyanin is unknown. No decision can be made as to whether or not the incorporated copper has to be bound specifically to a carrier protein. Furthermore, the role of the oxidation state of the copper remains open.

In an earlier study apostellacyanin [1], a representative for the type I Cu–protein [2], was successfully reconstituted using Cu–thionein. Because of the low redox potential near +180 mV compared to the +350 mV of Cu–thionein the Cu(I) transfer was possible both aerobically and anaerobically.

As the X-ray structure of plastocyanin is known [3] and the redox potential lies at +370 mV it was of interest to examine the transfer of Cu(I) into this high potential type I copper protein. In addition to Cu(I)–thionein its structural analogue $[Cu(I)(thiourea)_3]Cl$ [4] and the non-sulphur containing $[Cu(CH_3CN)_4]ClO_4$ were used. Circular dichroism was employed to monitor the Cu(I)-transfer directly and EPR measurements were carried out to distinguish between specifically and non-specifically bound Cu(II) following the oxidation with ferricyanide.

Experimental

All chemicals were of reagent grade quality and of commercial origin. Cu–thionein was isolated from baker's yeast [1, 5]. Plastocyanin was prepared from spinach using the procedure of Katoh [6]. Throughout Cu(I)–plastocyanin was obtained. After the oxidation with ferricyanide $A_{278} \times (A_{597})^{-1}$ was 2.5. The holoprotein was converted into the apoprotein after 7 h dialysis of 5 ml 0.1 m*M* Cu(I) plastocyanin against 50 m*M* KCN and 50 m*M* potassium phosphate buffer, pH 7.1 under argon. Excess cyanide was removed after repeated dialysis against 50 m*M* phosphate buffer. Crystalline $[Cu(I)(thiourea)_3]Cl$ and $[Cu(I)(CH_3CN)_4]ClO_4$ were synthesized as in [7, 8]. Copper was quantitated by atomic absorption spectrometry on a Perkin Elmer 400 S unit equipped with a HGA 76 B cuvette. EPR spectra were run on a Varian E 109 spectrometer and circular dichroism measurements were performed on a JASCO 20 A spectropolarimeter.

Results

Freshly prepared apoplastocyanin contained approximately 5% of the original copper. In the presence of $CuSO_4$ it was readily reconstituted to Cu(II)–plastocyanin. However, some extraneous copper was detectable which was removed after dialysis (Fig. 1). No blue colour was detected when an equimolar concentration of Cu–thionein was used. Upon the addition of a five fold molar excess of ferricyanide 15–20% of the holoprotein was reconstituted within seconds. Further reconstitution was substantially slower. More than 24 h were required to yield 70% of Cu(II)–plastocyanin. Unlike the

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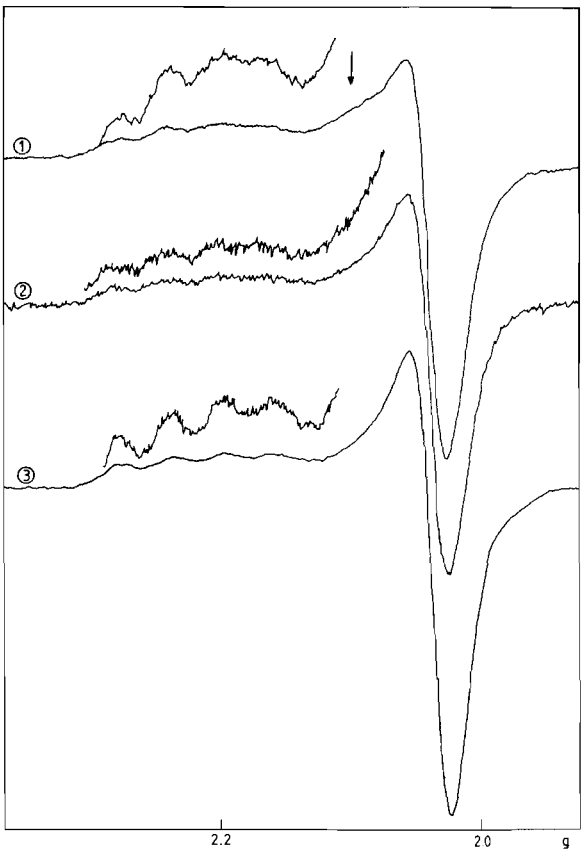

Fig. 1. EPR properties of differently reconstituted spinach plastocyanin. ① $CuSO_4$; $[Cu(I)(thiourea)_3]Cl$ or $[Cu(I)(CH_3CN)_4]ClO_4$ the latter two after oxidation with $K_3[Fe(CN)_6]$. Note the unspecific Cu(II) binding↓. ② Cu(I)–thionein and apoplastocyanin in the presence of $K_3[Fe(CN)_6]$ or the dialysed preparations of above. ③ Native plastocyanin. The concentration of plastocyanin and all copper donating compounds was 0.15 m*M*. $K_3[Fe(CN)_6]$ was 0.75 m*M*. Recording conditions: Temperature 77 K; microwave frequency 9.24 GHz; microwave power 12.5 mV; gain 2.5×10^3 in the inset 10^4.

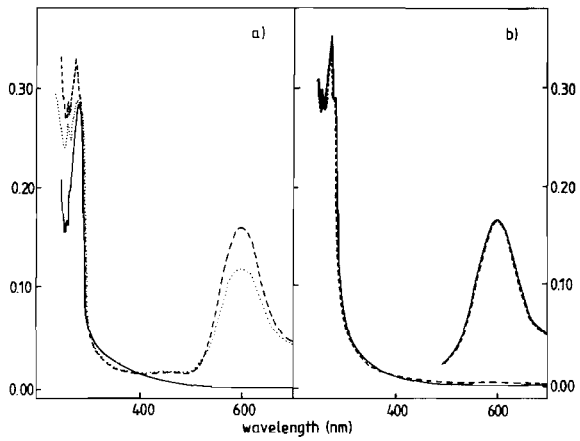

Fig. 2. Electronic absorption of differently reconstituted plastocyanin. a) (——) apoplastocyanin, (- - - -) native or $CuSO_4$ reconstituted plastocyanin, (· · · ·) Cu(I)–thionein + ferricyanide + apoplastocyanin. The Cu-concentration was 33 μM. b) (——) Cu(I)–plastocyanin (33 μM) from $[Cu(I)(thiourea)_3]Cl$ or $[Cu(I)(CH_3CN)_4]ClO_4$, (- - - -) native Cu(I)–plastocyanin. Inset Cu(II)–plastocyanins obtained after oxidation of the former Cu(I)–plastocyanins.

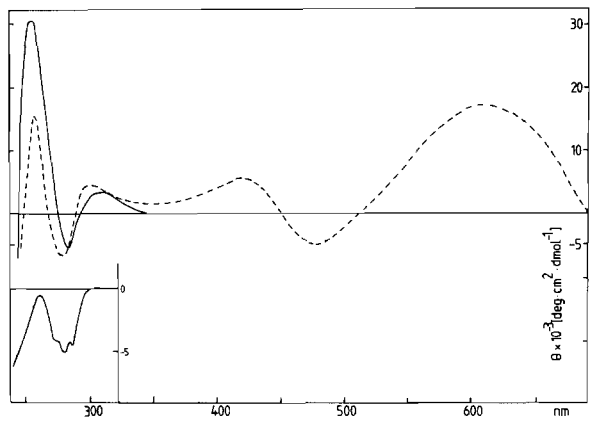

Fig. 3. Circular dichroism of (- - - -) native Cu(II)–plastocyanin, differently reconstituted and $[Fe(CN)_6]^{3-}$-oxidized Cu(I)–plastocyanin; (——) native and reconstituted Cu(I)–plastocyanin. Inset apoplastocyanin. For details see legends to Figs. 1, 2 and the text.

reconstitution with $CuSO_4$ no spurious Cu(II) was seen. The EPR properties were identical to curves 2 and 3 of Fig. 1. Unfortunately, circular dichroism measurements gave no evidence of a possible Cu(I)–plastocyanin. The overlapping signals of both Cu–thionein and plastocyanin did not allow any assignments.

Nevertheless, it remains a prominent task to examine whether or not Cu(I) can be transferred directly from a copper–thiolate chromophor into the specific metal binding site of apoplastocyanin. The structural analogues $[Cu(thiourea)_3]Cl$ and $[Cu(I)(CH_3CN)_4]ClO_4$ were employed to shed more light on this reconstitution problem.

These two Cu(I) complexes promised to be useful compounds to examine the electronic, magnetic and chiroptical properties of the active site reconstitution in more detail. No significant electronic absorption in the 600 nm region was seen when either Cu(I) complex was added to the apoprotein. Added ferricyanide developed the blue colour of Cu(II)–plastocyanin in an identical manner to that of the native oxidized Cu(II)–protein (Fig. 2). Again some unspecifically bound Cu(II) was

detected in the EPR spectrum (Fig. 1, curve 1) which was removed after dialysis.

The multibanded circular dichroism of Cu(II)–plastocyanin is in accordance with earlier work (for a review see [9]) (Fig. 3). Cu(I)-plastocyanin has no Cotton extrema in the visible region. A red shift of the 280 and 300 nm bands is seen. Concomitant with this shift the magnitude of the θ-values is diminished. The negative Cotton band at 280 nm of the apoprotein has two additional shoulders and no further band is seen below 300 nm. There is absolutely no detectable difference between the circular dichroism of native Cu(I)–plastocyanin and the respective Cu(I)–proteins reconstituted by $[Cu(I)(thiourea)_3]Cl$ or $[Cu(I)(CH_3CN)_4]ClO_4$. The direct incorporation of Cu(I) into the copper binding site is obvious. Oxidation with ferricyanide yielded the same Cotton bands as those usually obtained with native Cu(I)–plastocyanin.

Discussion

Unlike with stellacyanin [1] the Cu(I) transfer from Cu(I)–thionein into apoplastocyanin proceeds at a much lower rate. According to eqn. (1) the copper equilibrium appears to be located on the Cu–thionein side although the velocity of Cu(I) incorporation is expected to be very fast.

$$\text{Cu(I)–thionein} + \text{apoplastocyanin} \rightleftarrows \text{Cu(I)–plastocyanin} + \text{thionein} \quad (1)$$

$$\text{Cu(I)–thionein} + Fe[(CN)_6]^{3-} \longrightarrow \text{Cu(II)–thionein}_{ox.} \quad (2)$$

$$\text{Cu(II)–thionein}_{ox.} + \text{apoplastocyanin} \longrightarrow \text{Cu(II)–plastocyanin} + \text{thionein}_{ox.} \quad (3)$$

By way of contrast, the structural analogue $[Cu(I)(thiourea)_3]Cl$ is a perfect and complete Cu(I) donor to react with apoplastocyanin suggesting that this copper complex is either of low stability or more accessible to the copper binding site. Gassing of aqueous Cu(I)–thionein and $[Cu(thiourea)_3]Cl$ with H_2S resulted in the immediate precipitation of Cu_2S from the latter complex. Cu–thionein remained H_2S resistant for more than 10 h. The efficient Cu(I)-incorporation into apoplastocyanin can be attributed to the lower stability of the small M_r Cu(I)–$(SR)_4$ complex. When $[Cu(I)(CH_3CN)_4]ClO_4$ is used as a Cu(I)-donor complex some additional acetonitrile has always to be present for stabilization. In contrast, $[Cu(I)(thiourea)_3]Cl$ survives treatment with aqueous buffers for more than 24 hours. It should be used in Cu(I) reconstitution studies.

In the presence of ferricyanide the copper–thiolate chromophore of Cu(I)–thionein is oxidatively cleaved (2) leading to unspecifically coordinated Cu(II) in the polypeptide chain and cystine or even cysteic acid residues are formed [10]. The thermodynamic stability of this chelated Cu(II) is much lower compared to the tightly bound Cu(I)–thiolate binding centre with the consequence of the effective formation of Cu(II)–plastocyanin (3).

In conclusion incorporation of both Cu(II) and Cu(I) into plastocyanin is successful provided the stability constant of the Cu-donor compound is smaller compared to that of the holoprotein.

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