Dynamic Properties of the Minor Chlorophyll *a/b* Binding Proteins of Photosystem II, an *in Vitro* Model for Photoprotective Energy Dissipation in the Photosynthetic Membrane of Green Plants†

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Received October 18, 1995; Revised Manuscript Received November 21, 1995⊗

ABSTRACT: Excess light energy absorbed by the chloroplast membranes of green plants is dissipated by nonradiative de-excitation in order to protect against photodamage. This is observed as the nonphotochemical quenching of chlorophyll fluorescence, which has been suggested to result from an alteration in the structure and function of the chlorophyll *a/b* light-harvesting complexes of photosystem II (LHCII) due to the combined effects of protonation and the de-epoxidation of bound violaxanthin to form zeaxanthin. In agreement with this hypothesis, it is shown that the light-harvesting chlorophyll *a/b* proteins purified from spinach leaves exhibit pH-stimulated quenching of chlorophyll fluorescence; this quenching shares all the key features observed for the nonphotochemical quenching of chlorophyll fluorescence *in vivo*. In the case of the two minor complexes, LHCIIa (CP29) and LHCIIc (CP26), quenching is much greater than in the bulk complex LHCIIb and is strongly inhibited by the reagent dicyclohexylcarbodiimide. The carotenoids violaxanthin and zeaxanthin cause strong inhibition and stimulation of quenching, respectively, in these complexes. The results of this study are consistent with the suggestion that the minor light-harvesting complexes play a crucial role in photoprotective energy dissipation in the photosynthetic membrane of green plants. Moreover, for the first time, a system using isolated LHCIIa and LHCIIc for the study of the regulation of light harvesting is described.

When plants are exposed to light intensities in excess of those that can be used with maximum quantum yield, a dissipative process that removes the excess of absorbed energy is induced. This process, which is observed as the so-called ΔpH-dependent nonphotochemical quenching of chlorophyll fluorescence (qE),[1] is a mechanism that protects the photosynthetic apparatus, in particular the photosystem II reaction center, from photochemical damage (Horton & Ruban, 1992; Demmig-Adams & Adams, 1992). Two factors are associated with the induction of qE: the synthesis of zeaxanthin by de-epoxidation of the carotenoid violaxanthin (Demmig-Adams, 1990) and formation of the ΔpH (Briantais et al., 1979). There is general agreement that these two factors act synergistically to promote the state of maximum energy dissipation (Horton et al., 1991; Bilger & Björkman, 1994; Gilmore & Yamamoto, 1992), and spectroscopic data indicate that quenching occurs in the antenna of photosystem II [reviewed by Horton and Ruban (1994)], by means of the formation of a quenching complex (Gilmore et al., 1995). The presence of the xanthophyll cycle carotenoids in the light-harvesting chlorophyll *a/b* proteins (Peter & Thornber, 1990; Thayer & Björkman, 1992) lent support to other evidence that these proteins are the site of qE [reviewed in Horton et al. (1994)]. The fact that the major light-harvesting complex, LHCIIb, can be manipulated *in vitro* to give rise to fluorescence quenching is well-known, and it was proposed that qE occurs by the same mechanism in the LHCII system *in vivo* (Horton & Ruban, 1994). It was shown that acidification of purified LHCIIb gave rise to quenching that resembled qE in some detailed aspects (Ruban et al., 1994a): low pH and the presence of dibucaine stimulated quenching; whereas antimycin A caused inhibition, exactly similar to their effects on qE. Most significantly, addition of violaxanthin was found to inhibit quenching, while zeaxanthin was slightly stimulatory. Recently, examination of a range of carotenoids of different structure showed that all carotenoids with the number of carbon double bonds <9 had the same effect as violaxanthin, whereas if this number was >11, a stimulatory effect greater than that of zeaxanthin was found (Phillip et al., 1996). It was concluded from this work that the xanthophyll cycle carotenoids may exert both structural and photophysical changes upon LHCII. This conclusion is in part consistent with the model for qE that was derived from a detailed analysis of the regulation of quenching in isolated thylakoids (Horton et al., 1991). In this model, LHCII was proposed to be subject to allosteric control by protonation and by the binding of violaxanthin and zeaxanthin, with zeaxanthin preferentially binding to the protonated quenched state and violaxanthin binding to the deprotonated unquenched state. This model linked physiological observations of regulation *in vivo* to established principles of protein structure and function.

† Supported by Grant 50/C03100 of the Biotechnology and Biological Sciences Research Council of the United Kingdom and by a grant from the European Union Human Capital and Mobility Programme.

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⊗ Abstract published in *Advance ACS Abstracts,* December 15, 1995.

[1] Abbreviations: DCCD, dicyclohexylcarbodiimide; ΔpH, thylakoid proton gradient; DM, *n*-dodecyl *β*-maltoside; qE, ΔpH-dependent quenching of chlorophyll fluorescence.

0006-2960/96/0435-674$12.00/0

The LHCII system in plants is complex and consists of three minor complexes in addition to the LHCIIb. Recent work has called into question whether LHCIIb has the main role in qE and therefore cast some doubt on the significance of data obtained with this complex; instead, attention has focused upon two of the minor components of LHCII known as LHCIIa and LHCIIc (also referred to as CP29 and CP26, respectively) whose polypeptides are encoded by the *Lhcb4* and *Lhcb5* genes (Jansson, 1994). These proteins exhibit a high degree of structural homology with the major complex LHCIIb but are present in small amounts, approximately one polypeptide per PSII reaction center. Chlorophyll *b*-deficient mutants and plants developed in intermittent light (Jahns & Krause, 1992; Lokstein et al., 1994) which contain no LHCIIb show significant qE, supporting the involvement of the minor complexes. These complexes are also enriched in violaxanthin and zeaxanthin, binding one molecule per polypeptide compared to less than one per trimer for LHCIIb (Bassi et al., 1994; Ruban et al., 1994b), further supporting the suggestion that they might have an important role in qE. Moreover, it has been found that inhibition of qE in isolated chloroplasts by the carboxyl-modifying agent DCCD is associated with its covalent binding to LHCIIa and LHCIIc (Walters et al., 1994). Therefore, an explanation for qE is that protonation of these key carboxyl amino acid residues results in a structural change in either or both of these proteins, causing a change in pigment associations (Horton & Ruban, 1992, 1994) possibly by displacement of chlorophyll from glutamate binding sites (Crofts & Yerkes, 1994); quenching could result from either formation of a chlorophyll−chlorophyll dimer or by bringing chlorophyll into close proximity with a quenching carotenoid, zeaxanthin.

Up to now, there have been no studies of the behavior of the minor LHCII components *in vitro*; in order to test the applicability of the general principles of the LHCII model for qE, it is important to investigate whether LHCIIa and LHCIIc show a similar flexibility in photophysical properties as the widely studied LHCIIb. Similarly, it needs to be established whether those conditions known to be important for qE and which have been shown to affect LHCIIb have similar effects on the minor complexes. Moreover, it is important to discover if the suggested key role LHCIIa and LHCIIc in qE is expressed in a behavior of the purified complexes that is different from that shown by LHCIIb.

In this paper, we report the first results of an examination of isolated LHCIIa and LHCIIc. It is shown that these complexes have a large capacity for fluorescence quenching, greater than that of LHCIIb. The violaxanthin:zeaxanthin ratio and the pH synergistically control the fluorescence yield of both these proteins, and the effects of violaxanthin and zeaxanthin are much stronger than those observed for LHCIIb under identical conditions.

MATERIALS AND METHODS

The LHCII components, LHCIIa, LHCIIb, and LHCIIc, were isolated from dark-adapted spinach leaves by an isoelectric focusing procedure described previously (Ruban et al., 1994b). Negligible amounts of zeaxanthin and antheraxanthin are present in the complexes. The protocol for observation of quenching was exactly as described recently for the major complex, LHCIIb (Ruban et al., 1994a); briefly, LHCII solubilized in 200 μM *n*-dodecyl β-maltoside (DM) was diluted into 6 μM DM with constant stirring at a chlorophyll concentration of 2 μg/mL and a temperature of 20 °C. Chlorophyll fluorescence was monitored continuously using a Walz PAM fluorimeter, and quenching was quantified as the difference in fluorescence divided by the amplitude of the quenched fluorescence, $(F - F')/F'$, where F is the level of fluorescence recorded for a sample diluted into 200 μM DM. Acidification was achieved by addition of a small aliquot of HCl to give a range of pH down to pH 5.0. Purified violaxanthin and zeaxanthin dissolved in ethanol were added to a concentration of 10 and 20 μg/mL, respectively. Absorption and fluorescence spectra at 77 K were recorded using a SLM-Aminco DW2000 and an OMA-based instrument, respectively, described in detail before (Ruban & Horton, 1992).

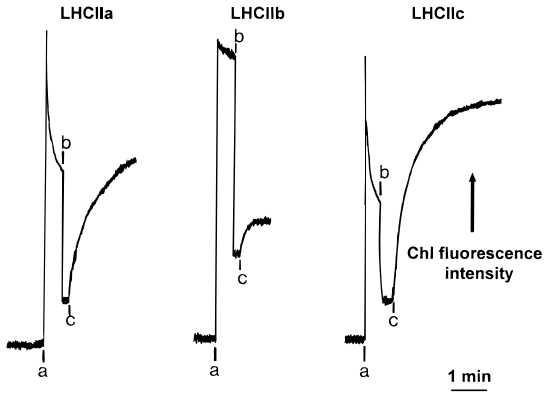

FIGURE 1: Fluorescence quenching in LHCIIa, LHCIIb, and LHCIIc upon dilution into 6 μM DM (a), acidification (b), and addition of DCCD (c).

Table 1: Quenching of Chlorophyll Fluorescence in LHCII[a]

treatment		quenching in LHCII $[(F - F')/F']$		
		LHCIIa	LHCIIc	LHCIIb
pH 7.8	control	1.4 ± 0.2	1.7 ± 0.3	0.02 ± 0.003
	dibucaine	3.8 ± 0.1	4.7 ± 0.1	1.0 ± 0.2
	antimycin	0.7*	0.8 ± 0.1	0.01 ± 0.004
pH 5.0	control	5.2 ± 0.9	7.8 ± 0.8	2.7 ± 0.3
	dibucaine	4.4 ± 0.5	6.2 ± 0.5	2.3 ± 0.2
	antimycin	4.8*	6.0 ± 0.4	0.8 ± 0.1

[a] Data were obtained as shown in Figure 1. The values are ± standard error obtained from 6−10 replicates, except for values marked with * which are single measurements. Antimycin (10 μM) and dibucaine (250 μM) were present in the dilution buffer. Quenching was calculated as $(F - F')/F'$ after 30 s (pH 7.8) and following acidification (pH 5.0), where F is the fluorescence intensity recorded for a sample diluted into 200 μM DM and F' is the quenched fluorescence level.

RESULTS

Figure 1 shows the response of LHCIIa, LHCIIb, and LHCIIc to quenching conditions *in vitro*; dilution into 6 μM DM at pH 7.8 results in a spontaneous quenching which slows down after approximately 10 s. This quenching was very small for LHCIIb but was significantly larger for LHCIIa and LHCIIc. Acidification to pH 5.0 then causes an immediate and strong acceleration of quenching for all complexes. The total final quenching was 5.2, 2.7, and 7.8 for LHCIIa, LHCIIb, and LHCIIc, respectively (Table 1).

It has been shown that the carboxyl-modifying agent DCCD is an inhibitor of qE in thylakoids, an effect associated with its binding to LHCIIa and LHCIIc, but not to LHCIIb,

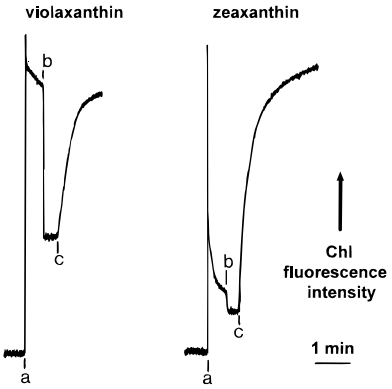

FIGURE 2: Effects of violaxanthin and zeaxanthin on fluorescence quenching in LHCIIa. The experiment was carried out as for Figure 1, with xanthophylls added prior to dilution.

this specificity being retained for DCCD labeling of isolated proteins (Walters et al., 1994). Quenching at low pH in LHCIIb was only weakly and irreproducibly inhibited by DCCD, but in contrast, there was substantial and consistent reversal of quenching in LHCIIa and LHCIIc following addition of DCCD (Figure 1). In fact, DCCD raised the fluorescence level above that found at pH 7.8. Other reagents know to strongly affect qE in thylakoids (Horton & Ruban, 1994) were tested. Dibucaine, which accelerates the formation of qE, stimulates quenching in all three complexes when present before acidification, but the final level at pH 5.0 was unaltered. Antimycin A, a strong inhibitor of qE, caused inhibition of both the spontaneous and low-pH-induced quenching for LHCIIb but was without effect on LHCIIa and LHCIIc (Table 1). A titration of the extent of the pH-dependent quenching for LHCIIb showed that the change occurred between pH 7.0 and 5.0 with an approximate p*K* of 6.2. Although detailed titrations of LHCIIa and LHCIIc have not yet been made, the enhancement of quenching occurred over a similar pH range (data not shown).

For LHCIIb, it has previously been shown that addition of violaxanthin inhibits the quenching of fluorescence in LHCIIb at pH 5.0, whereas zeaxanthin had either no effect (Ruban et al., 1994a) or a small stimulatory effect but only at pH 7.8 (Phillip et al., 1996). Much more dramatic effects of the carotenoids violaxanthin and zeaxanthin were observed with the minor complexes (Figure 2). Violaxanthin inhibited the quenching in LHCIIa at both pH 7.8 and 5.0. Addition of zeaxanthin causes a strong decrease in fluorescence intensity at pH 7.8, and a small amount of additional quenching was observed by acidification. The final extent of quenching was increased in the presence of zeaxanthin by about 20%, and all of the quenching observed in the presence of zeaxanthin was reversed by DCCD. Results similar to those observed for LHCIIa were also found with LHCIIc (not shown).

Quenching of LHCIIb *in vitro* has also been associated with formation of long wavelength emitters, particularly a 700 nm band at 77 K (Ruban & Horton, 1992). Emission spectra at 77 K also show a peak near 700 nm in the quenched state for the minor LHCII proteins (Figure 3). The spectra in Figure 3 were recorded under exactly the same conditions at equal chlorophyll concentrations; the reduced fluorescence intensity for the minor complexes compared to LHCIIb is consistent with the greater quenching at room temperature. The intensity of emission near 700 nm relative

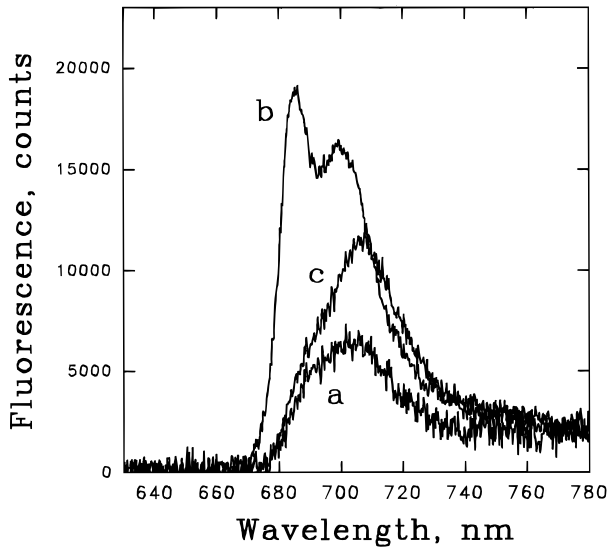

FIGURE 3: Fluorescence spectra at 77 K of LHCIIa (a), LHCIIc (c), and LHCIIb (b) in the fully quenched state obtained following acidification to pH 5.0.

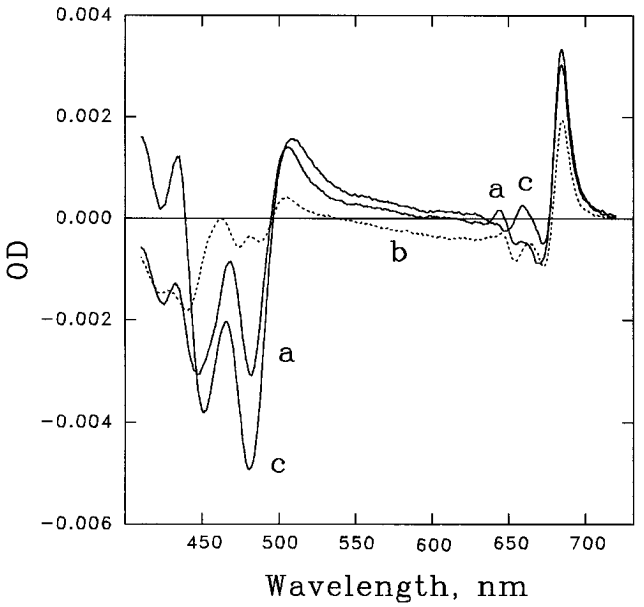

FIGURE 4: Absorption difference spectrum for quenched minus unquenched states of LHCIIa (a), LHCIIb (b), and LHCIIc (c). The unquenched sample was dissolved in 200 μM DM, whereas the quenched is that found at pH 5.0 in 6 μM DM.

to that near 680 nm is a characteristic index of aggregation-dependent quenching and is also in the order $c > a > b$, correlating with the extent of quenching at room temperature.

Quenching in LHCIIb is associated with changes in the absorption spectrum which have been shown not to result from a scattering artifact arising from the alteration in aggregation state (Horton & Ruban, 1994; Ruban et al., 1994a; Ruban & Horton, 1992; Jennings et al., 1994). Absorption difference spectra show similar features for all the LHCII components: negative bands at 440−450 nm and 480−490 nm arising from chlorophyll and xanthophyll, a positive band near 510 nm, and changes in the Q_y band of chlorophyll *a* and *b* (Figure 4). It is significant that the extent of the absorbance changes are larger for LHCIIa and LHCIIc than for LHCIIb, consistent with the larger quenching observed in these complexes. Particularly interesting is that the changes in xanthophyll absorption are much stronger for

the minor complexes than for LHCIIb, whereas the extent of the change in the chlorophyll *a* band near 685 nm is approximately in proportion to the differing extents of quenching in the three complexes. There is a 6−10-fold increase in the absorption change at 485 nm. The xanthophyll cycle carotenoid content is 3−4-fold higher in the minor complexes, suggesting that they are the origin of this absorbance change.

DISCUSSION

It has been shown that the fluorescence yield of isolated LHCIIa and LHCIIc can be manipulated *in vitro*; the extent of quenching is much greater than that previously shown for the major complex LHCIIb (Ruban et al., 1994a; Phillip et al., 1996), supporting the view that these complexes are the major sites of photoprotective energy dissipation in the photosynthetic membrane. Despite these important quantitative differences, the principal features of quenching in all the LHCII components are the same. The similarity of the absorption and fluorescence spectral changes indicates that the nature of the change in LHCII structure underlying the increase in quenching is similar for all the LHCII components. These changes are associated with increased protein−protein interactions, which have been shown in the case of LHCIIb to lead to the eventual formation of macromolecular aggregates (Ruban et al., 1994a) and result in dramatic changes in spectral properties of bound chlorophyll and xanthophyll. In addition to these absorption and fluorescence changes, linear dichroism spectra indicate increased excitonic interactions between chlorophyll *b* upon LHCII aggregation (A. V. Ruban, S. L. S. Kwa, R. van Grondelle, P. Horton, and J. P. Dekker, unpublished data) and evidence for changes in xanthophyll conformation and chlorophyll−protein H bonding has been obtained using resonance Raman spectroscopy (Ruban et al., 1995). However, so far it has not been possible to establish the nature of the quenching process that results from these alterations in pigment properties.

The specific conditions necessary for observing fluorescence quenching in isolated LHCII may resemble features of the membrane environment *in vivo*. In the case of the 10−50% lower fluorescence yield observed at pH 7.8 in 6 μM DM, this is most likely similar to the situation in the dark-adapted de-energized thylakoid membranes. Reduction in detergent concentration will expose the complex to water and promote protein−protein interaction. The process of quenching is probably the same as observed upon more drastic alteration in detergent concentration and resembles the aggregation process as discussed above. There is abundant evidence for strong pigment−pigment and protein−protein interactions within the LHCII system *in vivo* (Garab et al., 1988; Kolubayev et al., 1986; Bassi & Dianese, 1992). The fluorescence yield of PSII chlorophyll in the presence of closed reaction centers is much less than that of detergent-solubilized LHCII, which is close to that of free chlorophyll (Horton & Ruban, 1994). Similarly, in mutants lacking PSII reaction centers, the fluorescence yield is below that of a detergent-solubilized LHCII (Hodges & Moya, 1987). Thus, the LHCII *in vivo* in the dark exists in a partially aggregated, weakly quenched state. It is important to note that the quenching at pH 7.8 *in vitro* is inhibited by DCCD, suggesting that this quenching as well as that seen upon reduction of pH are due to the same underlying process. As discussed previously, there is a gradation of LHCII states from the highly fluorescent fully solubilized complex to the highly aggregated form; *in vivo*, qE operates over a fixed range within this continuum (Horton & Ruban, 1994).

When the ΔpH is formed across the thylakoid and qE is induced, there is a profound change in membrane properties, a decrease in thickness of approximately 20%, resulting in increased interaction between molecules and a change in polarity (Murakami & Packer, 1970). Such changes appear to result from protonation of LHCII as shown by the inhibitory effect of DCCD (Walters et al., 1994). The type of pH-dependent quenching in isolated LHCII described in this paper is therefore likely to be specific, and in fact, its characteristics strongly resemble qE in thylakoids. The spectral features of the absorption and fluorescence changes are similar for both quenchings (Horton & Ruban, 1994). The pH requirement for quenching in LHCII (p*K* 6.2) is similar to that observed for pH-dependent quenching in thylakoids (p*K* 5.7), and although the pH of the lumen in isolated thylakoids may be significantly lower than this, indirect estimates of the pH *in vivo* suggest a value much closer to that observed here for LHCII quenching (Pfündel & Dilley, 1992). Conditions which control qE have similar effects on *in vitro* quenching. Dibucaine stimulates qE and increases quenching in all LHCII components. Antimycin A, an inhibitor of qE, suppresses quenching in LHCIIb. Particularly important is that it has been shown here that DCCD inhibits quenching in LHCIIa and LHCIIc; DCCD covalently binds to these complexes in thylakoids when bringing about inhibition of qE (Walters et al., 1994). Most significantly, the quenching in LHCII is also controlled by the xanthophyll cycle carotenoids, namely, an inhibition and stimulation of quenching by violaxanthin and zeaxanthin, respectively. A dramatic stimulation of chlorophyll fluorescence quenching by zeaxanthin is shown for LHCIIa and LHCIIc, and the stronger effects observed with these complexes compared with those seen with LHCIIb provide further support for their key role in qE. The different effects of addition of zeaxanthin and violaxanthin to isolated LHCII are of course consistent with their well-documented role in the regulation of quenching *in vivo* (Demmig-Adams & Adams, 1992; Demmig-Adams, 1990).

It is important to note that low pH and zeaxanthin together do not lead to a significantly higher level of total quenching than does low pH alone. Similarly, zeaxanthin and low-pH-dependent quenching are both reversed by DCCD. It seems that violaxanthin and zeaxanthin respectively suppress or stimulate a quenching process within LHCII, for which there is an obligatory requirement for protonation exactly as for qE *in vivo*. The cooperative action of protonation and zeaxanthin/violaxanthin observed here for the minor LHCII is therefore consistent with the allosteric LHCII model for qE which proposes that the role of violaxanthin de-epoxidation to zeaxanthin is activation of quenching (i.e. lower the pH requirement) (Horton & Ruban, 1992; Horton et al., 1991, 1994) by stimulation of the formation of a quenched state of LHCII. However, the effects of this interaction between the xanthophyll cycle carotenoids and LHCII may be complex (Phillip et al., 1996) and could include both "direct" de-excitation of excited state chlorophyll by singlet−singlet interaction (production of zeaxanthin) (Owens, 1994; Frank et al., 1994) and indirect "anti-quenching" effects through control of protein structure (removal of violaxanthin) (Horton & Ruban, 1994). It is

possible that one of these effects could predominate over the other *in vivo*, although it is important to point out that it still has to be demonstrated that either of these effects on LHCII occurs when it is in the thylakoid membrane. Thus, an alternative explanation of the lower pH requirement for quenching in the presence of zeaxanthin *in vivo* may reside in the formation of the (more efficient) direct quenching process. Further experiments are required to directly test these hypotheses.

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BI9524878