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# Effect of $Nd^{3+}$ ion on carboxylation activity of ribulose-1,5-bisphosphate carboxylase/oxygenase of spinach ☆,☆☆

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**Abstract**

Neodymium (Nd), as a member of rare earth elements, proved to enhance the photosynthesis rate and organic substance accumulation of spinach through the increase in carboxylation activity of Rubisco. Although the oxygenase activity of spinach Rubisco was slightly changed with the $Nd^{3+}$ treatment, the specific factor of Rubisco was greatly increased. It was partially due to the promotion of Rubisco activase (R-A) activity but mainly to the formation of Rubisco–Rubisco activase super-complex, a heavier molecular mass protein (about 1200 kD) comprising both Rubisco and Rubisco activase. This super-complex was found during the extraction procedure of Rubisco by the gel electrophoresis and Western-blot studies. The formation of Rubisco–R-A super-complex suggested that the secondary structure of the protein purified from the $Nd^{3+}$-treated spinach was different from that of the control. Extended X-ray absorption fine structure study of the 'Rubisco' purified from the $Nd^{3+}$-treated spinach revealed that Nd was bound with four oxygen atoms and two sulfur atoms of amino acid residues at the Nd–O and Nd–S bond lengths of 2.46 and 2.89 Å, respectively.


*Keywords:* Nd; Rubisco; Rubisco activase; EXAFS; Spinach

Rare earth element (REE) fertilizers have been widely used in China for more than 30 years. It has been shown in many experiments that REEs could promote the growth of plants and increase the yield of crops [1–6]. The most important biological effect of REEs is their ability to promote the photosynthesis [5,7]. Our previous works revealed that $La^{3+}$, $Ce^{3+}$, and $Nd^{3+}$ could greatly increase the chlorophyll contents of spinach, accelerating the transformation from light energy to electron energy, the electron transport of PS II, water photolysis, and oxygen evolution [8–11].

Although many works have been carried out, the molecular mechanism of photosynthesis promotion by the REEs treatment still remains enigmatic. Moreover, the researchers mainly focused on the light chemical reaction part of photosynthesis. Few papers studied the effects of REEs on the carbon-linked reactions. Only Chen et al. once indicated that $LaCl_3$ could significantly promote the activities of tobacco Rubisco in vivo and in vitro, but the exact mechanism remains unclear [12]. Our previous researches,

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☆☆ *Abbreviations:* DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; Nd, neodymium; R-A, Rubisco activase; Rubisco, ribulose-1, 5-bisphosphate carboxylase/oxygenase; RuBP, ribulose-1,5-bisphosphate; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; PMSF, phenylmethanesulfonyl fluoride; $\Omega$, $CO_2/O_2$ specificity factor.
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however, found that the $La^{3+}$ and $Ce^{3+}$ treatment could result in the organic substance accumulation by promoting Rubisco activity. Though there was not enough experimental proof, it was assumed that a super-complex of Rubisco and Rubisco activase was formed [13].

Ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco, E.C.4.1.1.39) plays an important role as a determinant of biomass yield in photosynthesis. It is responsible for the photosynthetic carbon assimilation in catalyzing the reaction of carbon dioxide ($CO_2$) with ribulose-1,5-bisphosphate (RuBP) to form two molecules of D-phosphoglyceric acid (PGA). It also initiates photorespiration by catalyzing the reaction of oxygen, also with RuBP, to form one molecule of phosphoglycolate and PGA. Due to the importance of its carbon dioxide fixation in agriculture, forestry, environment, and its rather slow catalytic rate, Rubisco has received wide attention for its structure–function studies reviewed in Refs. [14–17]. It was found that the carbamylation of an active site lysine residue is essential to the activity of Rubisco [18]. The extent of this carbamylation depends on the concentration of $CO_2$ and $Mg^{2+}$; the dissociation of some phosphorylated compounds and RuBP from the noncarbamylated sites, particularly rely upon the activity of an enzyme called Rubisco activase (R-A). R-A was first revealed in the early studies of the rca mutant of *Arabidopsis* [19–21]. Since its discovery, a series of works have summarized the continuing research progress towards the understanding of the structure and function of this protein [22–24]. The protein, comprising two isoforms of 41–43 and 45–46 kD, is recognized to be a member of the $AAA^{+}$ family (ATPases associated with diverse cellular activases) whose members participate in the macromolecular complexes that perform the diverse chaperone-like functions [25]. As 'catalytic chaperone', R-A is thought to bind somewhere on Rubisco and induce a conformation change at the active site, which facilitates the dissociation of the inhibitory sugar phosphate from Rubisco. A super-complex model between Rubisco and Rubisco activase is therefore expected to be detected, though it has proven to be experimentally difficult [15,16,24].

Since the super-complex of Rubisco and R-A was found in the spinach treated with low concentration of $La^{3+}$ and $Ce^{3+}$ in our previous work, it is hypothesized that a suitable concentration of other REEs, such as $Nd^{3+}$, could also induce the formation of Rubisco–R-A super-complex in vivo [13]. The aim of the present study was to find the direct evidence of the Rubisco–R-A super-complex in vivo by the $Nd^{3+}$ treatment and further investigate the mechanism of REEs on increasing Rubisco carboxylation, which was not illuminated by any previous works.

## Materials and methods

*Physiology effect measurement. Spinacia oleracea* seeds were purchased from a local seed company and were soaked in 200 μmol $L^{-1}$ $NdCl_3$ solution at 15 °C for 48 h while the control group seeds were soaked in distilled water. Afterwards, the seeds were planted in $15 \times 15$ $cm^2$ pots without any fertilizer. Plants were grown in a growth chamber under 14 h of light (350 mmol photons $m^{-2}$ $s^{-1}$PAR) at 20 °C and 10 h of darkness at 15 °C/day, the relative humidity was 70%. The Nd group spinach in the stage of four leaves was sprayed once with 200 μmol $L^{-1}NdCl_3$ solution.

Seven days after spraying with 200 μmol $L^{-1}$ $NdCl_3$ solution, the fresh weight and dry weight of single plant were measured. The chlorophyll contents were determined according to Arnon's method [26], and the photosynthetic rate was detected with a portable photosynthesis detector of CI-301PS (CID Co., USA). Each index measurement was reduplicated for five times.

*Rubisco and Rubisco activase purification.* Approximately 100 g of spinach leaves was ground under liquid nitrogen in a chilled mortar, and the powder was homogenized with 200 mL of extraction buffer (100 mM BTP, pH 7.0, 5 mM $MgCl_2$, 1 mM EDTA, 1.4 mM ATP, 15 mM DTT, 1 mM PMSF, 2 mM benzamidine, and 0.01 mM leupeptin). From this material Rubisco and Rubisco activase were purified following the procedures reported by Sugiyama et al. and Salvucci et al., respectively [27,28]. The purified proteins analyzed by gel electrophoresis showed homogeneity; that is, only the two expected bands for each protein-55 and 14 kD for Rubisco, and 43 and 41 kD for R-A were observed. These results were similar to the ones obtained by the authors of the original reports. Protein concentration was determined by the method of Bradford [29].

*Enzyme activity assays.* Rubisco carboxylase activity assay procedure was performed by following Lan's method [30]. Modified protocol of Sivakumar et al. [31] was used for assaying oxygenase activity. The $\Omega$ values of Rubisco were also studied according to Jordon's methods [32]. The ATPase and Rubisco activation activity assays of Rubisco activase were performed as described elsewhere [33]. All the experiments were independently performed at 25 °C and the presented data are the average of the recordings from five independent experiments.

*Polyacrylamide gel electrophoresis.* Denaturing (SDS–PAGE) and nondenaturing polyacrylamide gel electrophoresis (native PAGE) were conducted as described by Laemmli [34]. In this work, 15% (w/v) resolving gel coupled to a 5% (w/v) stacking gel was utilized in SDS–PAGE, while 6% (w/v) resolving gel coupled to a 3% (w/v) stacking gel were utilized in native PAGE.

*Preparation of antibodies and Western-blot.* Polyclonal antibodies, recognizing Rubisco and Rubisco activase, were raised in New Zealand white rabbits as described by Sambrook and Fritsch [35]. For Western-blot, purified protein was electrophoretically separated on both SDS–polyacrylamide and native gel. Gels were either stained with Coomassie blue or blotted onto a nitrocellulose (NC) sheets as previously described [36]. The blot was incubated with anti-Rubisco antibodies or anti-Rubisco activase antibodies and then visualized by incubation with goat anti-rabbit IgG-HRP. The reaction bands were detected using DAB-stock stain.

*Assay of circular dichroism spectra of Rubisco.* Rubisco CD spectra were detected at 20 °C on a JASCO-J-810 spectropolarimeter with a quartz sample cell with a 1 cm optical path length. The concentration of protein samples was 0.1 mg $mL^{-1}$. Molecular ellipticities $[\theta]$ in deg $cm^2$ $dmol^{-1}$ were calculated using a mean residue weight of 114.2. The secondary structure indexes, α-helix, β-sheet, β-turn, random coil, disulfide bond, and aromatic amino acid contents of protein samples were determined by using Perczel's method [37].

*Assay of extended X-ray absorption fine structure (EXAFS) spectroscopy.* The Nd L(III) edge X-ray absorption spectra of 'Rubisco' purified from the $Nd^{3+}$-treated spinach were collected in the energy range from 6020 to 7300 eV in fluorescence mode under liquid nitrogen temperature at the 4W1B beam line of the Beijing Synchrotron Radiation Facility (operating at dedicated mode of 2.2 GeV and 80–40 mA). A Si(1 1 1) double-crystal monochromator was used and detuned to minimize the higher harmonic contamination at high energy region. Energies were calibrated using an internal Nd metal foil as standard. The first inflection of the edge of corresponding metal foil was used for energy calibration. The biological samples were placed in a cuvette and sealed with Kapton tape as transmission windows. A Lytle fluorescence detector was utilized with a Ti filter. Five scans were recorded and averaged in order to improve

the signal-to-noise ratio. No photon reduction was observed in comparison of the first and last spectra collected for a given sample.

The structural parameters were obtained by curve fitting the experimental data with the theoretical functions by nonlinear least-squares minimization of the residuals. The data were analyzed using the EXAFSPAK analysis suite (available from <http://ssrl.slac.stanford.edu/exafspak.html/>) together with theoretical standards from FEFF code [38], the latter used to calculate amplitude and phase shift functions.

## Results and discussion

### *Physiology effect indexes*

The physiology effects of spinach were greatly improved after $NdCl_3$ treatment (Fig. 1 and Table 1). As shown in Table 1, the single plant fresh weight and the dry weight of spinach with $Nd^{3+}$ treatment are increased by 54.11% and 80.77%, respectively; the chlorophyll contents are enhanced by 36.60%, the net photosynthetic rate promoted by 38.08% as compared with those in the control. The results indicate that $Nd^{3+}$, as well as other REEs, could significantly promote the growth of spinach, which is consistent with our previous studies [11]. Among all these increased indexes, the dry weight of plant increases most pronouncedly, indicating that the accumulation of organic substance of spinach could be greatly enhanced by $Nd^{3+}$ treatment. It should be closely related to the Rubisco, the key enzyme on the carbon dioxide fixation. Thus, the enzyme activity of Rubisco needs to be measured.


Fig. 1. Effect of $Nd^{3+}$ on the growth of spinach. The spinach was cultured as described in 'Materials and methods'. The picture was taken after four weeks cultivation.

Table 1
Effect of $Nd^{3+}$ on growth of spinach

| Indexes | Control | $Nd^{3+}$ |
|---|---|---|
| Single plant FW (g) | $1.46 \pm 0.22^a$ | $2.25 \pm 0.28^b$ |
| Single plant DW (g) | $0.52 \pm 0.06^a$ | $0.94 \pm 0.09^b$ |
| Chlorophyll content (mg $g^{-1}$ FW) | $1.53 \pm 0.14^a$ | $2.09 \pm 0.19^b$ |
| Net photosynthetic rate (μmol $CO_2$ $m^{-2}$ $s^{-1}$) | $19.25 \pm 0.73^a$ | $26.58 \pm 0.66^b$ |

The indexes listed above were determined by the assays described in 'Materials and methods'. The data presented are an average of the recordings from five independent experiments. a and b indicates that the differences of values followed by different letters in the same line are significant at $p = 0.05$ level ($t$ test).

### *Enzyme activity assay*

Rubisco behaves as a bifunctional enzyme, catalyzing carboxylation as well as oxygenation. Its bifunctionality stems from its lack of specificity between $CO_2$ and $O_2$ as substrates. The preference of Rubisco for $CO_2$ versus $O_2$ is represented by the specificity factor ($\Omega$), which is a constant of Rubisco for each species.

In order to prove the above assumption, we conducted the carboxylase and oxygenase activity measurement of the purified Rubisco from $Nd^{3+}$-treated and control spinaches (Table 2, the protein purification procedure was exactly the same). It could be concluded that the carboxylase activity of the $Nd^{3+}$-treated spinach Rubisco was extremely high, 2.39 times that of the control, while the oxygenase activity of the $Nd^{3+}$ group protein was almost equal to the control. The $\Omega$ value of two group enzymes was also measured. The control spinach Rubisco has a $\Omega$ value of 81, which is very close to that of the previous report [32]. However, the $\Omega$ value of the Nd group protein was 191 (Table 2), 135.80% higher than that of the control. It showed that the carboxylase capacity of Rubisco could be greatly enhanced by the $Nd^{3+}$ treatment. Moreover, the $CO_2$ fixation speed of the Rubisco at the atmospheric concentrations of $CO_2$ and $O_2$ began faster, which well explained the reason why $Nd^{3+}$ treatment could raise the organic substance accumulation of spinach.

As a molecular chaperon of Rubisco, the activity of R-A is a key limiting factor in regulating and maintaining the activity of Rubisco. The Rubisco activation activity assay of R-A was thus performed. As shown in Table 2, the Rubisco activation activity of R-A purified from the $Nd^{3+}$-treated spinach is 44.90% higher than that of the control.

Though five independent experiments lead to almost the same results, the promotion extent of the $CO_2$ fixation capacity of $Nd^{3+}$-treated Rubisco measured in the paper was a bit abnormally high. The R-A activity promotion

Table 2
Effect of $Nd^{3+}$ on the purified enzyme activities of spinach

| Indexes | Control | $Nd^{3+}$ |
|---|---|---|
| RuBP carboxylase activity (μmol $CO_2$ $mg^{-1}$ protein $min^{-1}$) | $2.46 \pm 0.01^a$ | $5.88 \pm 0.03^b$ |
| RuBP oxygenase activity (μmol $O_2$ $mg^{-1}$ protein $min^{-1}$) | $0.53 \pm 0.02^a$ | $0.51 \pm 0.02^a$ |
| Specificity factor ($\Omega$) | $81 \pm 2^a$ | $191 \pm 2^b$ |
| Rubisco activase activity (u $mg$ $protein^{-1}$) | $0.49 \pm 0.02^a$ | $0.71 \pm 0.02^b$ |

The indexes listed above were determined by the assays described in 'Materials and methods.' The enzyme activity data are values at 25 °C. The data presented are an average of the recordings from five independent experiments. a and b indicates that the differences of values followed by different letters in the same line are significant at $p = 0.05$ level ($t$ test).

from the $Nd^{3+}$ group was so limited that it could not be used to explain the phenomenon either. It suggests that the mechanism on the extremely high carboxylase capacity of the $Nd^{3+}$-treated Rubisco is very complicated and requires further investigation.

*Gel electrophoresis and Western-blot analysis*

To illuminate the mechanism of $Nd^{3+}$ on Rubisco activity, Rubsico and R-A were purified from spinach, separately, and assayed by SDS–PAGE, native PAGE electrophoresis, and Western-blot, and the results are shown in Figs. 2 and 3. Compared with the control Rubisco (Fig. 2A, lane 1), the 'Rubisco' purified from the $Nd^{3+}$ treatment group (Fig. 2A, lane 2) shows not only the two subunits of Rubisco at 55 and 14 kD but also two additional polypeptide strands at 41 and 45 kD. The same procedure was repeated several times, and all of them lead to the identical results. Since the control Rubisco under the same condition never showed the two additional polypeptides, it is believed that the two additional polypeptides could not be the degradation of Rubisco. Thus the questions have arose that what on earth are these two polypeptides. The R-A purified from the control spinach was also analyzed, and the result is shown in Fig. 2A (lane 3). The R-A protein comprised two isoforms at 41 and 45 kD, just the same as the above reports. More importantly, the molecular weight of the R-A subunits strands was very similar with the two additional polypeptides found in $Nd^{3+}$-treated 'Rubisco'. Therefore, the two additional polypeptides from $Nd^{3+}$-treated 'Rubisco' were thought to be the isoforms of R-A. Western-blot analysis further proved the assumption. Fig. 2B shows the Western-blot results with the antibody against Rubisco. Both the large subunits (LS, 55 kD) and small subunits (SS, 14 kD) of Rubisco in the two Rubisco lanes (control group and $Nd^{3+}$ group) could be revealed by the antibody against Rubisco, while the R-A lane (in lane 3) showed no protein print. When treated with the antibody against R-A, however, the two R-A isoforms as well as the two additional polypeptides in $Nd^{3+}$-treated 'Rubisco' could be clearly recognized (Fig. 2C). Both the LS and SS of Rubisco could not be displayed. The results strongly testified our deduction, that is, the 'Rubisco' purified from the $Nd^{3+}$ group contained R-A.

The native PAGE of the purified protein from the two groups was also studied. The control Rubisco lane only had one protein strand (Fig. 3A, lane 1), which was Rubisco holoenzyme with a reported molecular mass of 560 kD. As the linkage between the subunits would not break during the native PAGE procedure, R-A showed its oligomeric forms whose molecular mass was about 280 kD (Fig. 3A, lane 3). However, both a Rubisco holoenzyme strand and a heavier molecular mass protein strand were detected in the $Nd^{3+}$ 'Rubisco' lane, moreover, the heavier molecular mass protein strand could be revealed by both the antibody against Rubisco (Fig. 3B, lane 2) and the antibody against R-A (Fig. 3C, lane 2). According to Rubisco holoenzyme

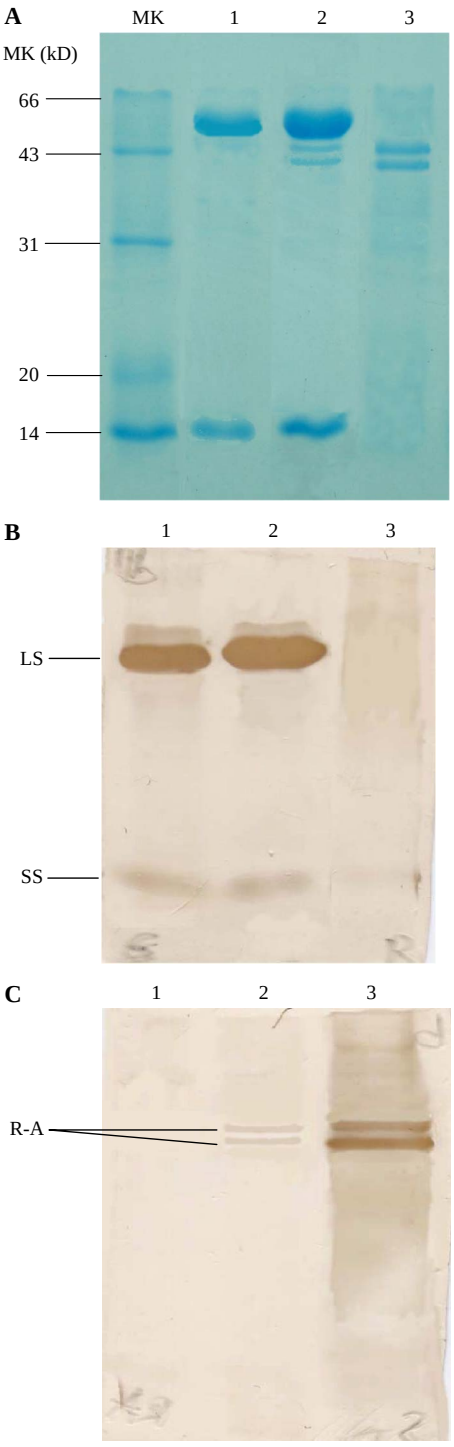


Fig. 2. Western-blot of SDS–PAGE. Purified protein, control spinach Rubisco (lane 1), $Nd^{3+}$-treated spinach Rubisco (lane 2), and control spinach Rubisco activase (lane 3) were resolved by SDS–PAGE (20 μg each). Gels were either stained with Coomassie blue (A) or transferred to nitrocellulose membranes for Western-blot analysis and revealed with antibodies raised against either Rubisco (B) or Rubisco activase (C). MK is for molecular mass markers. This panel is representative of three independent experiments.

print, the heavier molecular mass strand was estimated to be about 1200 kD. The pot experiments were replicated three times, and the results were all identical. They strongly

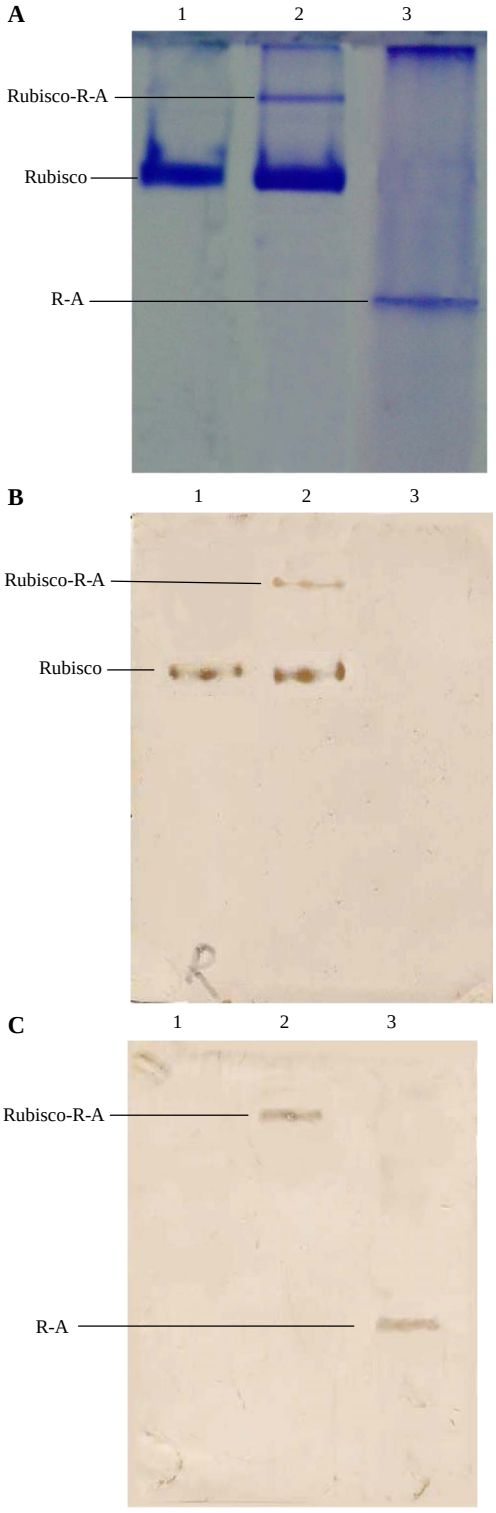


Fig. 3. Western-blotting of native PAGE. Purified protein, control spinach Rubisco (lane 1), $Nd^{3+}$-treated spinach Rubisco (lane 2), and control spinach Rubisco activase (lane 3) were resolved by native PAGE (20 μg each). Gels were either stained with Coomassie blue (A) or transferred to nitrocellulose membranes for Western-blot analysis and revealed with antibodies raised against either Rubisco (B) or Rubisco activase (C). This panel is representative of three independent experiments. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this paper.)

suggested that a super-complex of Rubisco and R-A was occurred in vivo after the $Nd^{3+}$ treatment. Another favorable proof about the formation of the super-complex had also been found. The 'Rubisco' purified from the $Nd^{3+}$ group demonstrates some ATPase activity (about 10% of the native R-A, data not shown here); which is the other enzyme activity of Rubisco activase. However, the Rubisco purified from the control group did not exhibit such ATPase activity.

Neuwald et al. [25] pointed out that R-A was a member of the $AAA^{+}$ family. To carry the function as 'catalytic chaperone', R-A was thought to bind somewhere on Rubisco and induce a conformation change at the active site, which facilitates the dissociation of the inhibitory sugar phosphate from Rubisco. Therefore, a super-complex model between Rubisco and R-A was detected, though the direct proof has not been obtained so far [15,16,24]. It was indicated, however, that R-A was chemically cross-linked to the large subunit of Rubisco and the co-immunoprecipitation of the two proteins was reported [39–41]. A preliminary electron microscopy study visualized a complex of Rubisco and activase specific for ATP-γ-S and $Mg^{2+}$, in which the activase seems to encircle the Rubisco holoenzyme [42]. Portis once supposed that in a Rubisco-R-A super-complex, eight active sites of Rubisco holoenzyme were enriched by 16 (or possibly 8) activase subunits [24]. Thus, the molecular mass of the Rubisco–R-A super-complex (1200 kD) found in our experiments is in good agreement with Portis's model. And the existence of the super-complex in the $Nd^{3+}$-treated spinach Rubisco could be the key reason for the extremely high promotion of the Rubisco carboxylase capacity by $Nd^{3+}$ treatment.

The super-complex is widely believed to occur in plants in vivo. However, our study on the control group spinach did not find the super-complex, consistent with other scientists' works. The reason might be attributed to the relatively slight amount of R-A (about 2% of soluble leave protein) and the extremely high amount of Rubisco (about 50% of soluble leave protein). This leads to a relatively low rate of activase/Rubisco, making the amount of super-complex of Rubisco–R-A in the control spinach too little to be detected [28]. Another reason may be attributed to the weak combination force between Rubisco and activase, so that the super-complex might partially dissociate during the purification procedure. On the contrary, $Nd^{3+}$ might enhance the expression of R-A (The purified protein contents from the $Nd^{3+}$-treated spinach were over 20% higher than those from the same weight of the control spinach, data not shown.), or induce the polymeric reaction of R-A, which would consequently raise the R-A to Rubisco ratio consequently. Otherwise, REEs might change the bound kinetics properties of the activase, thus promoting the combination between Rubisco and activase. This assumption needs to be testified in near future.

*CD spectra assay*

Secondary structure alteration of the purified proteins from $Nd^{3+}$-treated spinach was further studied by using CD spectroscopy (Fig. 4). The CD spectra of the control Rubisco have negative Cotton peaks at 208 and 218 nm, which is consistent with the reports on rice and spinach [43,44]. The CD spectra of the purified enzymes from $Nd^{3+}$-treated spinach, however, are quite different from that of the control. The secondary structure indexes of the purified enzymes were studied by fitting them with five standard structure spectra. Tomimatsu and Miao's results show that the α-helix contents in spinach Rubisco, rice, and tobacco are, respectively, 32%, 47%, and 30% [43,44]. We considered the contribution of β-sheet, β-turn, random coil, disulfide bond, and aromatic amino acid residue to $[\theta]$ value, which were not calculated in Tomimatsu and Miao's results. The α-helix contents in Rubisco of control spinach in the paper were 42%. However, the α-helix in the protein from $Nd^{3+}$ treated spinach was calculated to be 48.7%, while the β-sheet and random coil contents were 6% and 21.8% lower than those of the control, respectively. The β-turn and disulfide bond and aromatic amino acid residue contents were 9% and 12.1%, respectively, higher than those of the control. The increased amounts of disulfide bonds and aromatic amino acid residues of the $Nd^{3+}$-treated group protein were also in some way proved the conformation difference between the two groups of purified proteins.

*EXFAS assay*

X-ray absorption spectroscopy (XAS) has been proved to be a very powerful technique to probe the local structure around specific elements. The extended X-ray absorption fine structure (EXAFS) contains information on local atomic arrangement about each individual type of absorber atom as described in the theoretical formula based on the single scattering contribution to EXAFS. The development of X-ray fluorescence excitation XAS enables the detection of low concentrations of transition metals present in metalloenzyme systems. Chen studied the radical distribution function of La in pure Rubisco [12]. It was found that La was bound to the oxygen atoms of Rubisco at the distances of the La–O bond of 2.69 and 2.513 Å, respectively, and the third shell may contain either sulfur (S) or phosphorus (P) atoms at 4.6 Å. It had also been proven that RuBPcase provided some ligands (amino acid residues) to be intimately involved in the catalysis, and the divalent cations, $Mg^{2+}$ and $Ca^{2+}$, were bound with the oxygen atoms of Lys201, Asp203, and Glu204 of Rubisco large subunits, with the Ca–O bond distance of 2.3–2.6 Å and the Mg–O bond of 2.23–2.37 Å. Thus, Chen believed that the Rubisco binding with $La^{3+}$ is different from that with $Mg^{2+}$; the oxygen atoms at 2.69 Å may originate from $H_2O$ molecules, while the other three oxygen ligands may come from Lys201, Asp203, and Glu204 of Rubisco large subunits, respectively [45,46].

Did Nd cause the frame alteration of the protein directly or just make some indirect influence? In order to investigate the formation mechanism of the Nd induced Rubisco–R-A super-complex, we employed X-ray absorption techniques to study the coordination structure at Nd sites. Strong Nd L(III) edge spectra were detected from the 'Rubisco' purified in $Nd^{3+}$-treated spinach, indicating that Nd was already bound to the 'Rubisco'. The Fourier transform for the $k^3$-weighted Nd L(III) edge EXAFS oscillations in the range of 2–14 $Å^{-1}$ and the scattering path contributions obtained from curve fittings are shown in Fig. 5. The local structure coordination parameters obtained from the curve fitting are listed in Table 3. It showed that Nd was bound with four oxygen atoms on the protein in its first shell at the distance of the Nd–O bond of 2.46 Å. The second shell at 2.89 Å may be either

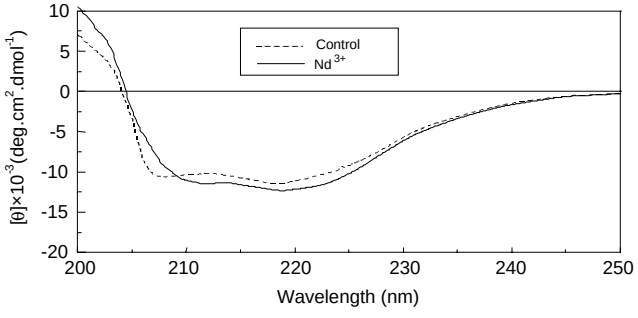


Fig. 4. Effect of $Nd^{3+}$ on ultraviolet CD spectra of the purified protein from spinach. The experimental conditions were exactly followed as given in 'Materials and methods'. The curve was representative of ten independent scans.

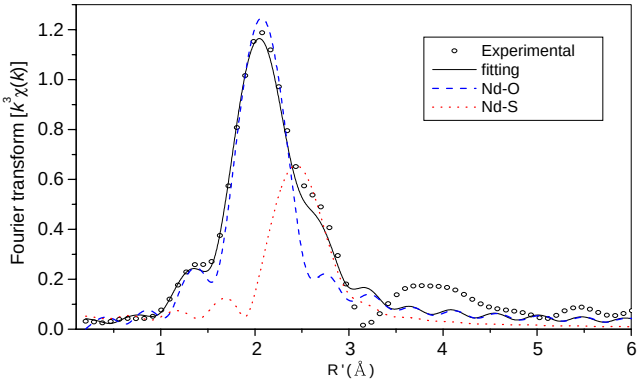


Fig. 5. Radical distribution function of $Nd^{3+}$ in the purified protein from spinach. Radical distribution function (Fourier transforms of $k^3$-weighted EXAFS oscillation $\chi(k)$) of $Nd^{3+}$ in the purified protein from spinach. The experimental curve (open circles) is compared with the fitting result (solid black line). The nonlinear least-square curve fitting revealed that Nd is bound with four oxygen atoms on purified protein in its first shell (blue) at the distance of the Nd–O bond of 2.46 Å. The second shell (red) around 2.89 Å may be either sulfur (S) or phosphorus (P) atoms. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this paper.)

Table 3
The coordination parameters at Nd sites obtained from EXAFS curve fitting

| Shell | Ligand numbers | $R$ (Å) | $\sigma^2$ (Å$^2$) | $\Delta E_0$ (eV) |
|---|---|---|---|---|
| Nd–O | 4 | 2.46 | 0.0029 | 3.72 |
| Nd–S (P) | 2 | 2.89 | 0.0037 | |

The errors of data and fits are roughly estimated from the change of the residual factors to be 15% for $N$, 0.25% for $R$, 10% for $\sigma^2$, and 4 eV for $\Delta E_0$. No ambiguities of the theoretical standards are included. Shell indicates the type of ligands for each shell of the fit. Atom numbers are the coordination numbers, $R$ is the metal-scatterer distance. $\sigma^2$ is a mean square deviation in $R$. $\Delta E_0$ is the shift in $E_0$ for the theoretical scattering functions.

sulfur (S) or phosphorus (P) atoms. The Nd–O and Nd–S bond lengths in the Rubisco–R-A complex fall into the typical ranges of 2.46–2.58 and 2.85–2.95 Å, respectively, according to the CSD (Cambridge Structure Database) investigation. Obviously, our results are different from the above reports. It might be attributed to the formation of Rubisco–R-A super-complex. It also suggested that Nd might be bound with the oxygen atoms of amino acid residues or the sulfur atoms of sulfhydryl groups. The Nd atoms were thought to be a 'bridge' between the Rubisco and R-A, which could consequently make the structure of super-complex tighter.

## Conclusion

Because of their special physical and chemical characteristics, REEs were found to be able to greatly enhance the light energy absorption, energy transfer, electron transport rate, and photophosphorylation rate, which consequently promotes the organic substance accumulation of plant. However, it also has a close relation to the enzyme activity of Rubisco. As revealed in our previous works, though the extraction procedures of Rubisco are the same, the protein purified from the $Nd^{3+}$-treated spinach is greatly different from that of the control. An additional heavier protein concomitant with the Rubisco purification product from the $Nd^{3+}$-treated spinach was found, which was testified to be a super-complex of Rubisco–R-A by gel electrophoresis and Western-blot analysis. Therefore, the secondary structure of the protein was changed and the carboxylase activity of Rubisco was consequently promoted.

From the wide variety of Rubisco structures now available, it could be found that the minimal conformation differences exist between the carbamylated and uncarbamylated structures [47]. In contrast, more dramatic conformational differences exist in the structures containing bound sugar phosphates [48], which basically fall into two alternative states corresponding to either an 'open' or 'close' active site [46,47,49]. A key process in the proposed activase mechanism is that after the activase forms a complex with Rubisco, it catalyzes the movement of the amino-terminal domains toward the position seen in the open structures. Since Nd atoms were found to bind with the oxygen atoms of amino acid residues or the sulfur atoms of sulfhydryl groups on the protein complexes, it was supposed that Nd atom might favor the physical interaction between Rubisco and its catalytic chaperon, Rubisco activase, making the 'open' Rubisco structure steadier. However, the $\Omega$ values of the $Nd^{3+}$-treated spinach Rubisco were already proven to increase, and the carboxylase capacity of Rubisco was greatly improved.

Unfortunately, the super-complex found by these experiments had a relative low quantity, thus the pure super-complex of Rubisco–R-A has not been isolated yet due to the technical limitations. All that was found was regretfully the binary complex of Rubisco and super-complex of Rubisco–R-A. However, the paper could in some way help us to clarify the Rubisco's $CO_2$ assimilation ability and find out the role of Nd for its interesting biological functions, which in turn would serve the practical uses in agriculture.

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