Q_A Binding to D2 Contributes to the Functional and Structural Integrity of Photosystem II

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Z. Naturforsch. **45c,** 359–365 (1990); received November 3, 1989

Photoinhibition, Plastoquinone, Photosynthesis, Site-Directed Mutagenesis, Cyanobacteria

Two D2 mutants were created with a site-directed mutation near the presumable binding site of Q_A. In one of the mutants, in which Trp-253, the aromatic residue potentially involved in facilitating electron transport from pheophytin to Q_A and/or in binding of Q_A, had been replaced by Leu, PS II was undetectable in thylakoids. This mutant is an obligate photoheterotroph. In another mutant the Gly-215 residue, located next to the His residue that is proposed to bind Q_A and Fe^{2+}, was mutated to Trp. This mutation leads to a rapid inactivation of oxygen evolution capacity in the light, and to a virtual elimination of the potential to grow photoautotrophically, but does not greatly affect the number of photosystem II reaction centers on a chlorophyll basis. We propose that proper binding of Q_A to the photosystem II reaction center complex is a prerequisite for stability of the photosystem II complex. Impairment of Q_A binding leads to rapid inactivation of photosystem II, which may be followed by a structural disintegration of the complex.

Introduction

The photosystem II (PS II) complex is composed of a number of polypeptides, two of which create the binding environment for redox-active cofactors involved in PS II electron transport [1, 2]. These two polypeptides, D1 and D2, have regional sequence homology to the reaction center proteins of photosynthetic purple bacteria [3, 4]. In view of the facts that there is a striking functional homology between PS II and the reaction center of photosynthetic purple bacteria [5, 6], and that a high-resolution three-dimensional structure of the reaction center from two species of purple bacteria has been determined [7, 8], a number of hypotheses has been developed regarding the structure of the PS II reaction center and the function of specific amino acid residues in D1 and D2. In particular, models identifying ligands to the reaction center pigment P680, to the non-heme iron between the plastoquinones Q_A and Q_B, and to Q_A were developed on the basis of homologies with the bacterial system [9, 10].

To experimentally test such models, and to determine the sensitivity of the PS II complex towards changes in ligands and in the environment thereof, a procedure was developed to introduce site-directed mutations in the *psb*D gene, coding for D2, and to have the modified gene expressed in the cyanobacterium *Synechocystis* sp. PCC 6803 for comparison of PS II phenotype in mutant and wild type [11–13]. This cyanobacterium is a facultative photoheterotroph (it can be propagated in the absence of PS II activity when glucose is supplemented to the medium), is spontaneously transformable, and can incorporate the foreign DNA into its genome by homologous recombination (reviewed in [11, 12, 14]). These properties are fundamental to the site-directed mutagenesis procedure involving deletion of the wild-type *psb*D genes from the cyanobacterium, and replacement by a *psb*D copy carrying a site-directed mutation [13].

The D2 protein is known to harbor D, a PS II donor identified to be the Tyr-160 residue in D2 [15, 16], and, on the basis of homologies with the reaction center from purple bacteria, is thought to contribute to binding P680 and the non-heme iron as well as to create most of the binding pocket of Q_A and a pheophytin [2, 4, 5]. This paper will concentrate on the region of D2 presumed to contribute to binding of Q_A, and on the role Q_A binding may play in creating a functionally active and structurally stable PS II complex.

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Verlag der Zeitschrift für Naturforschung, D-7400 Tübingen
0341–0382/90/0500–0359 $ 01.30/0

Extrapolating from the structure of the reaction center from purple bacteria [7, 17], the Q_A-binding environment is thought to be constituted of several parts of the D2 protein, principally the region of the fourth putative membrane-spanning helix near the stromal side of the thylakoid, including His-214 (amino acid numbering for cyanobacteria; is homologous to His-215 in plants), and a region of the interhelical sequence between the putative membrane-spanning helices 4 and 5 (including Trp-253, which is Trp-254 in plants). From site-directed mutagenesis of the His-214 residue it had been concluded that this residue was crucial to structural integrity of the PS II complex [18], thus suggesting that binding of Q_A is important for a stable assembly of PS II.

To extend the investigation towards the role of Q_A binding on PS II structure and function, the effects of mutations in other residues in the putative Q_A-binding environment were determined: site-directed mutations were introduced in Gly-215, the residue next to His-214, and in Trp-253. It was observed that such mutations had a significant impact on overall PS II structure and function, suggesting that in PS II Q_A not only plays an important role as one of the electron transport intermediates, but also contributes to the stabilization of a functional PS II complex.

Materials and Methods

Growth and transformation of Synechocystis

Growth conditions of wild type and mutants have been described previously [19]. The procedure to generate a "double-deletion" mutant of *Synechocystis* (lacking the *psb*DI/C operon as well as *psb*DII, the second copy of the D2 gene) has been outlined in [13]. A site-directed mutation was introduced into the cyanobacterium by transformation with a plasmid carrying a single-codon change in the *psb*DI/C operon at the desired location (see below) along with a kanamycin-resistance cartridge [13]. Transformants were identified by their resistance to kanamycin, purified, and characterized.

Site-directed mutagenesis

The method utilized for oligonucleotide-directed mutagenesis in *psb*DI cloned into M13 mp 18 has been described [13]. The oligonucleotides used were 5′ CACCGTGGCCCAGTGGATAGC 3′ to introduce the G215W mutation, and 5′ CTGAGACAAGAAACG 3′ for the W253L mutation. The mismatches with the complementary wild-type sequence have been underlined. Introduction of *psb*DI/C carrying the appropriate site-directed mutation into the *psb*DI/C/*psb*DII$^-$ double-deletion mutant of *Synechocystis* was carried out essentially as described in [13].

Mutant characterization

Oxygen evolution measurements on wild type and mutant cells were performed on a Gilson oxygraph, model KM. Actinic light was provided by an Oriel Xenon Arc lamp (model 6143). The light was filtered through 15 cm water, and through a Schott OG-570 filter before reaching the sample. The incident light intensity was 7000 $\mu E \cdot m^{-2} \cdot s^{-1}$, and was saturating for a maximal rate of electron transfer with 0.5 mM $K_3Fe(CN)_6$ as electron acceptor; 2,6-dimethyl-*p*-benzoquinone (DMQ) (0.1 mM) was added to mediate electron transfer between thylakoids and the non-penetrating $K_3Fe(CN)_6$. In addition to the electron acceptors, the medium for O_2 evolution measurements in intact cells contained 25 mM N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid (HEPES)/NaOH, pH = 7.2. The chlorophyll concentration used for oxygen evolution assays was 10 μg/ml.

Quantitation of PS II on a chlorophyll basis in whole cells was done by [^{14}C]diuron binding. Various concentrations (5–100 nM) of radiolabeled diuron (243 μCi/mg; Amersham) were added to 1 ml samples containing cells at 25 μg/ml chlorophyll in 25 mM HEPES, pH = 7.2. "Control" samples in addition contained 20 μM unlabeled atrazine, to measure the amount of diuron that is bound to cells to other sites than to PS II, and that cannot be competed off by an excess of atrazine. After incubation 15 min at room temperature in dim light, cells were pelleted by centrifugation in a microcentrifuge, and 0.8 ml of the supernatant was mixed with scintillation cocktail and counted in the scintillation counter. The amounts of free and specifically-bound diuron were calculated for each of the concentrations of added diuron.

For the preparation of thylakoids from wild type and mutant *Synechocystis*, a procedure modified from that described by Burnap *et al.* [20] was used. Ten liters of late-log phase cells grown in

BG 11 with 5 mM glucose were harvested by centrifugation, and washed in 50 mM sodium phosphate (pH = 7.0). The cells were resuspended into 10 ml of thylakoid buffer containing 50 mM HEPES (pH = 7.0), 5 mM $MgCl_2$, 50 mM $CaCl_2$, 5% (v/v) glycerol, and 0.5% (v/v) dimethylsulfoxide (DMSO). The suspension was incubated on ice for 1–2 h to allow equilibration, and after centrifugation the cells were resuspended into approximately 30 ml of the same buffer, and mixed with an equal volume of 0.1 mm glass beads. Cells were broken in a Braun homogenizer by three 30 s bursts at the maximum speed setting. Cell debris, unbroken cells and glass beads were pelleted by centrifugation at low speed ($1000 \times g$; 4 min), and the supernatant was centrifuged again at $15{,}000 \times g$ for 15 min. The pellet, containing larger thylakoid fragments, was resuspended in thylakoid buffer, and, after its chlorophyll concentration had been determined, was frozen in liquid N_2 and stored at −85 °C. Methods used for SDS-polyacrylamide gel electrophoresis and Western blotting have been described by Vermaas *et al.* [21].

Results

Two D2 mutants were generated, G215W and W253L, with a mutation of Gly-215 to Trp, and Trp-253 to Leu, respectively. These mutations are in residues in putative proximity to Q_A. The two mutants were analyzed with respect to their PS II properties.

Growth characteristics

To determine the photoautotrophic competence of the two mutants, growth of wild type and mutants was monitored as the optical density of the culture at 730 nm (representing scattering by cyanobacterial cells) under photoautotrophic and photoheterotrophic conditions. Fig. 1 shows that the photoautotrophic growth rate of G215W is poor (the strain does not significantly grow under such conditions, but does not die either), whereas the W253L mutant does not show PS II-dependent growth at all and bleaches out gradually. In the presence of glucose, when PS II activity is not necessary for growth, the growth rate is similar in wild type and mutants, indicating that indeed PS II activity has been affected in the two mutants.

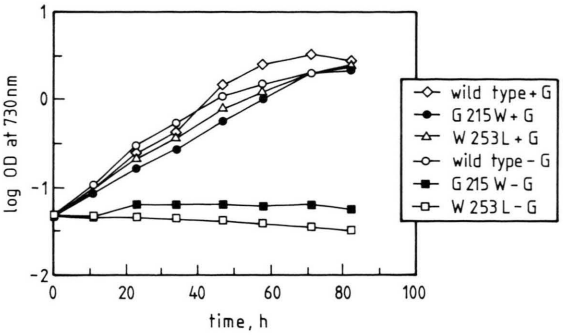

Fig. 1. Growth curve of wild type *Synechocystis* sp. PCC 6803 and mutants G215W and W253L in the presence and absence of glucose (G). A decrease in optical density in time is indicative of cell death. To introduce linearity in the initial phase of growth, on the vertical axis the logarithm of the optical density has been plotted.

Quantitation of PS II in the thylakoid

A convenient and efficient method to determine the amount of PS II in the thylakoid in intact cells is to measure the amount of a radiolabeled PS II-directed herbicide that binds to cells and that can be replaced by another unlabeled PS II-directed herbicide. From measurements at different concentrations of labeled herbicide, both the dissociation constant of the herbicide and the number of herbicide-binding sites on a chlorophyll basis can be determined [22]. Fig. 2 shows a double-reciprocal plot of [^{14}C]diuron-binding data obtained with wild type and G215W. No specific diuron binding could be detected in W253L. The data have been corrected for non-specific binding of diuron by subtracting the diuron binding that occurs in the

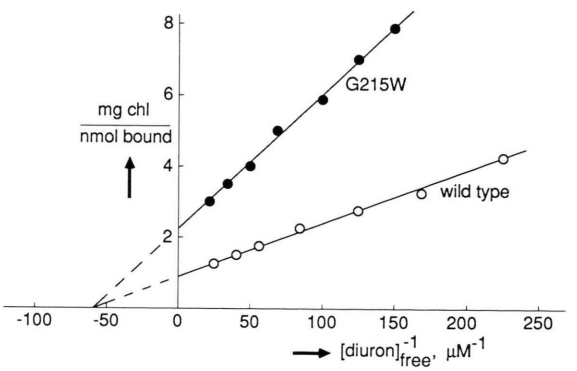

Fig. 2. Double-reciprocal plot of [^{14}C]diuron binding to wild type and G215W cells. 25 μg chlorophyll per ml.

presence of 20 μM unlabeled atrazine. The intersect with the *X*-axis represents the negative inverse of the diuron dissociation constant, whereas the intersect with the *Y*-axis represents the maximum amount of herbicide that can be (specifically) bound on a chlorophyll basis. Assuming that there is one high-affinity diuron-binding site per PS II, and taking the molecular weight of chlorophyll *a* (the only chlorophyll species present in this cyanobacterium) to be 894, there is one PS II per 1000 chlorophylls in wild type; in G215W there is about 40% PS II on a chlorophyll basis as compared to wild type, whereas in W253L there is no significant specific diuron binding. Note that the PS II/chlorophyll ratio can vary from experiment to experiment (*cf.* Table I). However, this ratio is consistently a factor of 2.0–2.5 higher in wild type than in G215W provided that the cyanobacterial cultures were grown up in parallel and were harvested at approximately equal cell density.

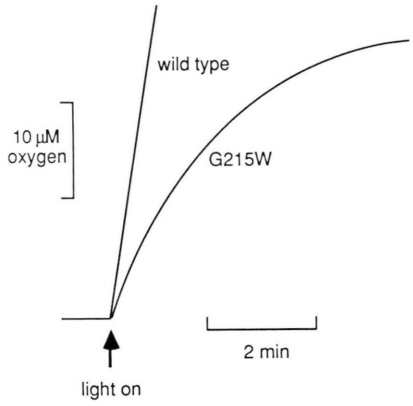

Fig. 3. Oxygen concentration in wild type and G215W cell suspensions as a function of time. The medium contained 25 mM HEPES, pH = 7.5, 0.5 mM $K_3Fe(CN)_6$ and 0.1 mM 2,6-dimethyl-*p*-benzoquinone. 10 μg chlorophyll per ml. The initial rates of oxygen evolution were 218 and 87 μmol $O_2 \cdot (\text{mg chlorophyll})^{-1} \cdot h^{-1}$, respectively. After 1 min of illumination, the wild type rate has remained unchanged whereas the rate of oxygen evolution in G215W has declined to 54 μmol $O_2 \cdot (\text{mg chlorophyll})^{-1} \cdot h^{-1}$.

Table I. Diuron binding in wild type *Synechocystis* sp. PCC 6803 and G215W cells after illumination. Light intensity: 7000 $\mu E \cdot m^{-2} \cdot s^{-1}$.

Strain	Light [min]	Chlorophyll/ binding site	%	K_D [nM]
Wild type	0	550	100	15
	1	625	88	16
	3	750	73	16
	5	950	58	18
G215W	0	1200	100	16
	1	1650	73	16
	3	2800	43	16
	5	4500	27	17

Oxygen evolution measurements

The Hill reaction in wild type and mutant cells was measured as oxygen evolution in continuous saturating light. As presented in Fig. 3, in G215W a rapid decline in the oxygen evolution rate was observed after the onset of illumination. Within approximately 5 min, the oxygen evolution rate in G215W had decreased to zero, whereas in wild type the oxygen evolution was very stable. In W253L, no Hill reaction was observed at all (not shown).

Protein composition

To investigate whether the altered properties of PS II in the mutants are reflected in changes in the presence of the reaction center proteins D1 and D2, Western blots of thylakoid proteins from wild type and mutants were probed with D1 and D2 antisera (not shown). There was no D1 or D2 detectable in W253L, whereas the PS II reaction center proteins are present in thylakoids of G215W (see Fig. 4), in amounts roughly comparable to those in wild type.

"Photoinhibition"

From the data presented thus far, it can be concluded that G215W contains a relatively normal amount of PS II in its thylakoids, but that the PS II activity decreases rapidly in saturating light. To investigate whether this PS II inactivation could be correlated with a loss of PS II reaction center proteins or with a loss of structural integrity of the PS II reaction center at large, the amounts of D1 and D2 as well as the diuron-binding characteristics were measured before and after illumination in saturating light under conditions similar to those described in the legend to Fig. 2. Fig. 4 shows that the amount of D1 and D2 in the thyla-

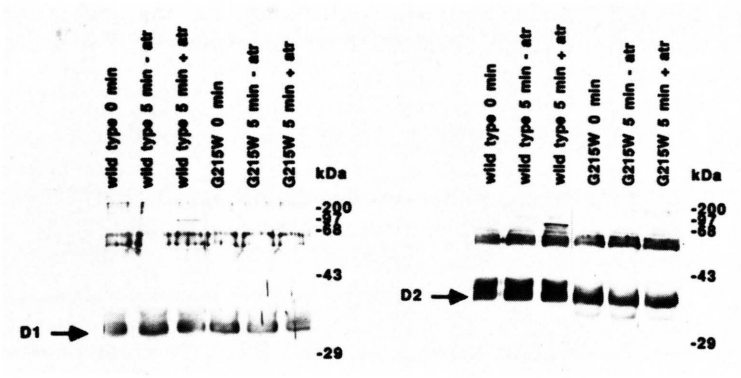

Fig. 4. Detection of D1 and D2 in thylakoids isolated from wild type and G215W. Before thylakoid isolation, the cell sample of each strain was divided into three parts. One part was kept in the dark ("0 min"), one part was illuminated with strong light (7000 $\mu E \cdot m^{-2} \cdot s^{-1}$) in the presence of ferricyanide and dimethyl-*p*-benzoquinone for 5 min under conditions identical to those used in Fig. 3 ("5 min − atr"), and one part was also illuminated, but 20 μM atrazine was added before the start of illumination ("5 min + atr").

koids as determined by Western blotting in the G215W mutant is somewhat lower than in wild type (in accordance with the results shown in Fig. 2), and more importantly, that the amount of the reaction center proteins in the thylakoid do not significantly decline upon illumination. The addition of atrazine before illumination (shown to protect thylakoids from light-induced D1 turnover) also does not have an effect on the amounts of D1 and D2. It should be kept in mind, however, that there is not necessarily a linear relationship between the amount of staining of the Western blot and the amount of antigen; thus, the results shown in Fig. 4 should not be interpreted to indicate that there is no degradation of the reaction center protein at all, but rather that there is a significant amount of these proteins left under conditions where there is no oxygen evolution detectable anymore.

It is perhaps of importance to note that there is a band on the Western blot cross-reacting with the D2 antiserum at a position below the normal D2 band in G215W, but not in wild type. A faint cross reaction of D2 antisera with a very small polypeptide (of 4000−8000 M_r) has also been observed in G215W; however, the intensity was too low to show in the photograph. It is noteworthy that the apparent breakdown product of D2 in G215W is present before and after illumination, and is therefore not directly related to the inhibition of oxygen evolution in the light.

The bands on the Western blot corresponding to proteins of 60,000 M_r or more can, in part, be ascribed to oligomers of D1 and/or D2. Such bands are routinely observed on Western blots of thylakoid proteins probed with D1 and D2 antisera.

The results of the herbicide-binding experiments after illumination have been tabulated in Table I: in G215W, illumination results in a somewhat faster decay of the number of diuron-binding sites than in wild type, but the loss in the number of binding sites is slower than the loss of oxygen evolution. The diuron dissociation constant does not change appreciably in either wild type or mutant.

Discussion

The results presented in this paper indicate that two residues, G215 and W253, presumed to be close to the Q_A-binding site in PS II, cannot be altered without changing the functional integrity of the PS II complex. A similar conclusion had been reached for H214N, a mutant in which the His residue presumed to be involved in Q_A/Fe^{2+}-binding has been mutated [18]. In contrast, mutations in the W250 residue of the M subunit of *Rhodobacter capsulatus* (which is the homolog of W253 in *Synechocystis* D2) do not appear to have such a significant effect on stable reaction center assembly, since in such mutants reaction centers can still be observed even though Q_A is no longer bound [23].

Since in purple bacteria a mutation of Trp-250 to Leu in the M subunit impairs Q_A binding to the reaction center [23], it is reasonable to postulate that in PS II a similar Q_A binding impairment may occur in W253L. In view of the fact that both W253L and H214N do not contain a significant amount of PS II in their thylakoids, it is attractive

to assume that the function that has been impaired in these mutants (*i.e.*, binding of Q_A) is a crucial factor towards the stabilization of PS II and/or expression of *psb*D. This implies that, in contrast to the situation in purple bacteria, Q_A is required for stability within the reaction center complex.

This extends the results of Diner and coworkers [24], who observed that upon extraction of Q_A from a PS II preparation from *Chlamydomonas reinhardtii* the reaction center complex enters a metastable state: rapid reassociation of the quinone with PS II restores primary charge separation, whereas reaction center complexes that do not rapidly reassociate with quinone irreversibly lose their ability for primary charge separation, even when quinone is added back. This is in contrast to the situation in purple bacteria, where quinone can be easily extracted from the Q_A site, leaving a stable reaction center which can be reconstituted to a functionally active entity by a number of different quinones.

The results obtained with G215W appear to indicate that in this mutant Q_A binding can occur (a significant amount of PS II reaction centers capable of binding diuron is present in the thylakoid), but that upon light treatment electron transfer through PS II is progressively lost. It should be stressed that this does not immediately result in a structural rearrangement leading to a loss of diuron-binding sites and of the reaction center proteins: the kinetics of the latter two processes are significantly slower than that of the deactivation of oxygen evolution. Thus, in this mutant photoinhibition primarily is a progressive inhibition of electron transport upon illumination, and is not triggered by turnover of one of the reaction center proteins. Degradation of D1 and D2 are effects rather than causes of photoinhibition.

The primary site responsible for the progressive inhibition of electron transport upon illumination has not yet been identified. However, since Gly-215 is next to the His residue presumed to be a ligand to Q_A and the non-heme iron it is reasonable to postulate that the site of photoinhibition in this mutant may be at the Q_A site. Several lines of evidence support this hypothesis. In the first place, Q_A binding appears to be crucial to functional and structural integrity of the PS II complex as the results on the H214N and W253L mutants imply. Moreover, recently, double-reduction and subsequent release of Q_A has been suggested to be a possible factor contributing to photoinhibition [25, 26]. Since G215 is presumed to be close to the Q_A-binding site, a change of the Gly residue to a bulky Trp residue can be easily imagined to cause a change in Q_A binding and/or a modification in the redox properties of this plastoquinone, giving rise to inhibition of electron transport in the light, possibly due to physical loss of Q_A.

A potential problem with the hypothesis that Q_A is directly involved in photoinhibition is that the primary protein target for degradation is not D2, but the D1 protein (which is less closely associated with Q_A than D2 is). In G215W D2 appears to be relatively unstable (judging from the apparent breakdown product observed in Fig. 4), even in darkness. However, D1 degradation products cannot be observed by Western blotting in this mutant. Another problem with the hypothesis regarding Q_A involvement in photoinhibition is that this hypothesis does not account for the fact that PS II inhibitors such as atrazine protect from photoinhibition, unless one adopts an *ad hoc* hypothesis that atrazine or other herbicides affect the binding and/or redox properties of Q_A.

However, it should also be pointed out that it is unlikely that the Q_A region is the only site of photoinhibition in PS II. Similar light-dependent inhibition has been found to occur in mutants that are impaired in electron transport at the PS II donor side [27]. Thus, it is entirely possible that photoinhibition is not primarily caused by events occurring at one particular site, but that multiple causes can lead to the same effect.

An important point that may need additional discussion is the significant difference in phenotype between the virtually PS II-less W253L, and the rapidly photoinhibited, but PS II-containing G215W. A possible reason for this difference is that the affinity of Q_A for its binding site is different in the two mutants. In W253L, the Q_A affinity may be so low that no quinone binding takes place at the Q_A site, causing a rapid turnover of the PS II complex; in *Rhodobacter capsulatus* a mutation of the homologous W to L in the M subunit also leads to a loss of Q_A affinity. In G215W, binding of Q_A may occur with reasonably normal affinity, thus allowing a relatively normal amount of PS II complexes to accumulate in the thylakoids. Upon reduction of Q_A during illumination, it is possible that PS II function in G215W gradually decreases

because of a progressive loss of Q_A, which may be caused by either a relatively low affinity of Q_A^- for its binding site in the mutant as compared to in wild type, or to a double-reduction and subsequent loss of Q_A. Other mutations are introduced in the Gly-215 residue to help identify the mechanism by which PS II activity is lost upon illumination.

It is obvious that site-directed mutations around the putative Q_A site in PS II have a number of interesting effects of PS II function, structure and stability. However, at this moment it is not yet possible to go much beyond wild speculation on the potential physiological significance of the Q_A site on PS II stability and structure. Nonetheless, it is hoped that further mutagenesis in this region of the D2 protein will provide additional information regarding the function of Q_A in the structure and stable assembly of PS II.

Acknowledgements

This research has been supported by the National Science Foundation (DCB87-16055). This is publication # 47 from the Center for the Study of Early Events in Photosynthesis. The Center is funded by U.S. Department of Energy grant DE-FG02-88ER13969 as part of the Plant Science Center program of the U.S. Department of Agriculture/Department of Energy/National Science Foundation.

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