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Characterisation of photoinduced breakdown of the D1-polypeptide in isolated reaction centres of Photosystem II

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When the isolated reaction centre of Photosystem II, reconstituted with the quinone, 2,5-dibromo-3-methyl-6-isopropyl-*p*-benzoquinone (DBMIB), is exposed to photoinhibitory illumination, a D1-polypeptide breakdown product of 24 kDa is detected by immunoblotting. In addition, weaker bands are also detected at 17, 13 and 10 kDa. It is suggested that the 24 kDa D1-polypeptide breakdown product is the same as that first observed in vivo by Greenberg et al. (1987) EMBO J. 6, 2865–2869. Its appearance in isolated Photosystem II reaction centres requires the presence of an electron acceptor, but occurs under both aerobic and anaerobic conditions. In our in vitro experimental system the photoinduced degradation of the D1-polypeptide to the 24 kDa fragment was related to the functional activity of the reaction centre and the enzymatic nature of the proteolysis was characterised by a pH optimum of about 8.0 and by inhibition with proteinase inhibitors, especially the serine-type soybean trypsin inhibitor. The results support our earlier findings (Shipton and Barber (1991) Proc. Natl. Acad. Sci. USA 88, 6691–6695) that the appearance of the light-induced D1-polypeptide breakdown pattern of fragments occurs as a consequence of donor side photoinhibition when highly oxidising species accumulate in the reaction centre and bring about pigment oxidation and degradation. We suggest that it is this selective loss of pigments that induces a conformational change in the D1-polypeptide which triggers its autoproteolytic cleavage.

Introduction

Recently, our knowledge of the relationship between the photochemical damage and the triggering mechanism that brings about the degradation of the D1-polypeptide of Photosystem II (PS II) has grown. The cleavage of the D1-polypeptide has been found to be proteolytic and can be separated from the photoinduced damage. Indeed, Aro et al. [1] showed that D1-polypeptide degradation can occur in the dark after a preillumination period at low temperatures, as long as the temperature is raised. It has been argued that the primary site of proteolytic cleavage is close to the alpha-helix destabilizing stretch of amino acids, rich in glutamate, serine and threonine, located between putative transmembrane segments IV and V of the D1-polypeptide [2]. In agreement with this proposal, studies with intact systems have detected a cleavage product at 23.5 kDa [3,4]. A breakdown product of similar size has also been detected when isolated thylakoids and PS II cores were exposed to strong illumination [1,5]. Although the precise origin of the 23.5 kDa fragment has recently become a matter of debate (Ref. 6 and Cook, M. and Barber, J., unpublished data), studies with the above in vitro systems have shown that the proteinase responsible for the photoinduced degradation of the D1-polypeptide is located in a stoichiometric amount within the PS II core complex [5]. This conclusion has been taken further in our work with the isolated reaction centre of PS II, consisting of only the D1- and D2-polypeptides, the subunits of cytochrome *b*-559 and the 4.8 kDa product of the *psb*I gene. We have shown that this minimal PS II reaction centre complex still retains the capacity to bring about the proteolytic cleavage of the D1-polypeptide [8]. In this

Abbreviations: Chl, chlorophyll; DBMIB, 2,5-dibromo-3-methyl-6-isopropyl-*p*-benzoquinone; FTIR, Fourier transform infrared; Hepes, *N*-2-hydroxyethylpiperazine-*N'*-ethanesulphonic acid; Mes, 2-(N-morpholino)ethanesulphonic acid; PMSF, phenylmethanesulphonyl fluoride; Tricine, *N*-tris (hydroxymethyl)methylglycine; SDS-PAGE, sodium dodecyl sulphate polyacrylamide gel electrophoresis

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paper we report further experimental work to characterise this autoproteolytic capacity of the isolated PS II reaction centre.

Materials and Methods

The D1–D2/cytochrome *b*-559 reaction centre of PS II was isolated from pea thylakoid membranes. The procedure used was a modification of that first reported by Nanba and Satoh [9] and has been detailed by Chapman et al. [10].

Photoinhibitory light treatments were performed in a stirred glass cuvette for various times and temperatures as indicated in the figure legends. For such treatments the reaction centre concentration corresponded to a level of 50 μg ml^{-1} chlorophyll and the normal suspension medium was 50 mM Tris plus 2 mM dodecyl maltoside at pH 8.0 (except where indicated). In the experiment designed to test the pH optimum of the breakdown of the D1-polypeptide we utilized a range of buffers, all at a concentration of 50 mM. At pH 5.0, 5.5, 6.0 and 6.5 Mes buffer was used, Hepes buffer for pH 7.0 and 7.5 and Tricine at pH 8.0 and 8.5. Dodecyl maltoside was added at the usual concentration of 2 mM. Heat-filtered (Schott filter KG1) white light (6000 μE m^{-2} s^{-1}) was generated by an incandescent Flexilux 650 lamp. The electron acceptor, DBMIB, was used at a concentration of 200 μM. In the experiment of Fig. 2, anaerobic conditions were achieved by the addition of 10 mM glucose, 0.2 mg ml^{-1} catalase and 0.2 mg ml^{-1} glucose oxidase, and the cuvette was repeatedly flushed with nitrogen prior to illumination. The proteinase inhibitors phenylmethanesulphonyl fluoride (PMSF), antipain, E-64 and phenanthroline monohydrate (Sigma) were all used in an equimolar concentration with the reaction centre.

Photochemical activity of the PS II reaction centres was measured in two ways: (1) charge separation was followed as the reduction of silicomolybdate (250 μM) using 1 μg ml^{-1} Chl and 1 mM $MnCl_2$ as an electron donor. This absorption change was followed at 500 nm. A blue-green filter (Schott BG18) protected the photomultiplier and a red cut-off filter (Schott RG665) provided the actinic light; (2) reduction of cytochrome *b*-559 was followed as an absorption increase at 559 nm with 4 μM DBMIB and 1 mM $MnCl_2$ as a donor to P680. PS II reaction centres were present at a chlorophyll concentration of 3 μg ml^{-1}. All absorption measurements were performed on a Perkin-Elmer 557 spectrophotometer at 10°C.

10 to 17% polyacrylamide gradient gels containing 6 M urea were used for analysis of the polypeptide composition of samples. Gels were prepared for immunological assays using western blotting [11]. Profiles of separated polypeptides were electrophoretically transferred onto nitrocellulose and detected using rabbit primary antibodies to the D1-polypeptide from pro-

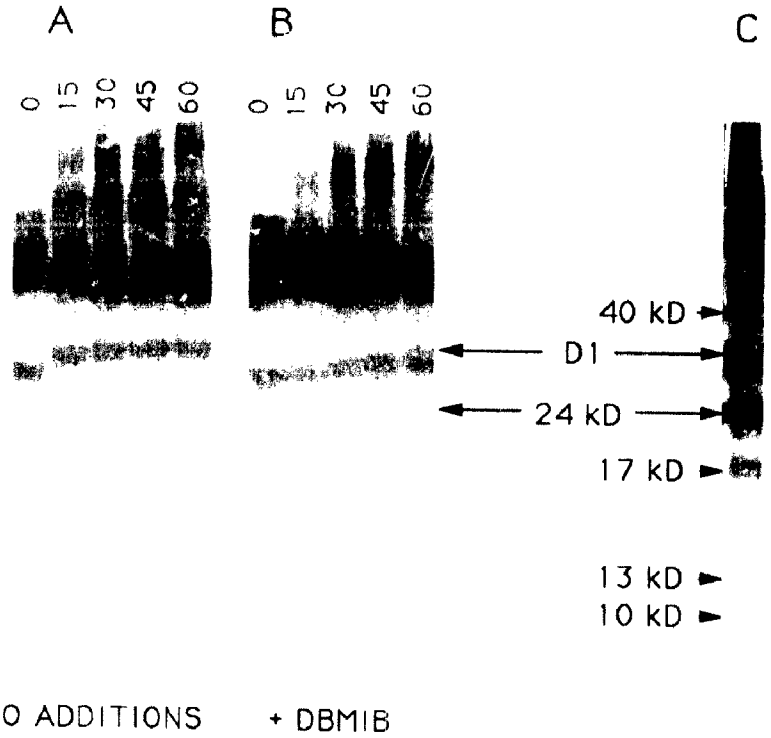

Fig. 1. (A) + (B) Show western blots with α-D1 antiserum of a photoinhibitory treatment time course of the PS II reaction centre in the absence and presence of the electron acceptor DBMIB, respectively. Light treatments were performed for 15, 30, 45 and 60 min at 20°C. (C) presents a western blot of a lane similar to (B) (60 min) to show all breakdown products detected by α-D1 antiserum as described previously [24].

tein generated by expression of the *psbA* gene in *E. coli* [12]. As shown in Ref. 12, this polyclonal antibody showed a high specificity to the D1 polypeptide and did not cross-react with other PS II proteins. Alkaline phosphatase conjugated secondary antibodies (Sigma) were then employed, followed directly by colour development using the appropriate chromogenic substrates. Time-courses carried out for the purpose of comparison were run on the same gel and blotted onto one sheet of nitrocellulose which could then be treated in a uniform manner throughout the western blotting procedure. Western blots were scanned to quantify the formation of the 24 kDa major breakdown fragment, using a Shimadzu gel scanner (CS-930).

Results

Fig. 1A shows that in buffer containing no added electron acceptor, the exposure of the isolated PS II reaction centre to photoinhibitory light leads to a gradual loss of the D1-polypeptide with no obvious appearance of breakdown products. It seems that under these conditions the photoinduced loss of D1-polypeptide in the 30 kDa region is due to aggregation as detected by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) using Coomassie blue staining [13]. However, when DBMIB is present as an electron acceptor during the illumination, the loss of D1-polypeptide is associated with the appearance of a D1-polypeptide fragment having an apparent molecular mass on SDS-PAGE of 24 kDa (Fig. 1B). Other lower-molecular-weight D1-polypeptide breakdown products, at about 17, 13 and 10 kDa, are also present but are more difficult to visualise (Fig. 1C). The dark broad band observed at about 50 kDa in Fig. 1 is artifactual and not consistently seen in our western blots using the same D1-polypeptide antibody. The origin of this artifact is unknown but relates to the methodology of our immunological analyses. In this experiment it partially masks the position of the D1/D2 heterodimer and also makes it difficult to detect any aggregated states. The appearance of the photoinduced D1-breakdown fragments with DBMIB present is very consistent and has been reported previously for isolated reaction centres [8]. Also similar-sized D1-polypeptide breakdown products have been observed when isolated thylakoids [1] or PS II cores [5] were subjected to strong illumination. In the case of the isolated PS II reaction centres, we have shown previously that the artificial electron acceptor silicomolyb-

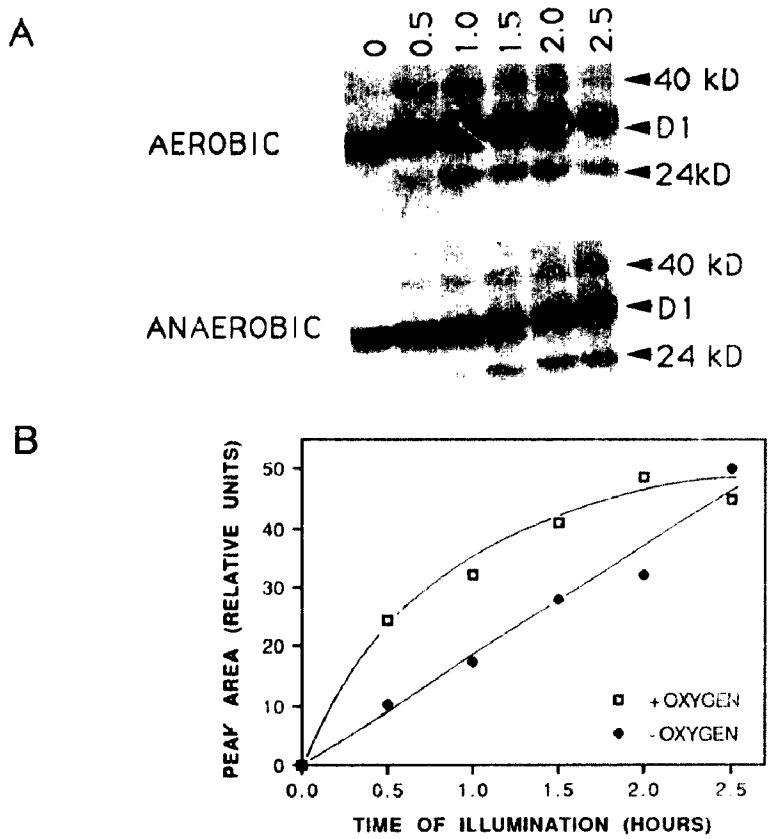

Fig. 2. (A) A western blot with α-D1 antiserum showing time courses of photoinhibitory treatment of the reaction centre under aerobic and anaerobic conditions. Illumination was performed for 0.5, 1.0, 1.5, 2.0 and 2.5 h at 20°C. Samples were taken for solubilization at each time point. (B) Shows quantification of those data by densitometer scanning of the western blot.

date can replace DBMIB [8] ; s can decylplastoquinone (data not presented) in order to obtain the breakdown pattern shown in Fig. 1B and C. Note that western blotting also detected a weak band at about 40 kDa of unknown origin [8]. A common feature of this type of experiment is that with increasing time of illumination the non-degraded D1-polypeptide gradually increases in apparent molecular mass on SDS-PAGE. This modification occurs whether an electron acceptor is present or not and probably relates to conformational changes of the type detected by FTIR spectroscopy [14].

The level of the 24 kDa breakdown fragment was found to be related to the light intensity of the preillumination (data not shown), and it could easily be detected after 30 min of illumination by intensities comparable to full sunlight (1000–2000 μE m^{-2} s^{-1}). In contrast, the cleavage of the D1-polypeptide to the 24 kDa fragment was not absolutely dependent on the presence of oxygen, although the rate of appearance of this fragment was slightly retarded under anaerobic conditions (Fig. 2). It has also been observed that illumination in the presence of an electron acceptor and a free radical scavenger (propyl gallate, 500 μM) causes no retardation in the appearance of the 24 kDa breakdown product (data not shown). We therefore conclude that the difference between anaerobic and aerobic conditions probably relates to O_2 acting as an electron acceptor and competing with the quinone-dependent cytochrome *b*-559 protective cycle [8].

Fig. 3 gives the results of an experiment designed to test whether degradation of D1 to the 24 kDa fragment

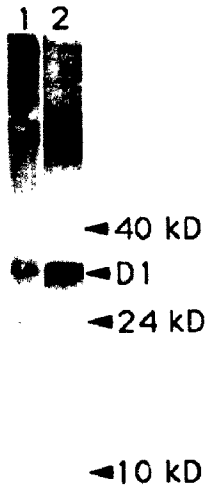

Fig. 3. Lanes 1 and 2 show a western blot for D1-related polypeptides in the PS II reaction centre after 30 min of photoinhibitory illumination in the presence of 200 μM DBMIB at 20°C. The sample shown in lane 2 had, prior to this treatment, been illuminated without an acceptor or donor with light at an intensity of 6000 μE m^{-2} s^{-1} to decrease its photochemical activity by 50% (as measured by $Mn^{2+} \rightarrow$ silicomolybdate electron transport).

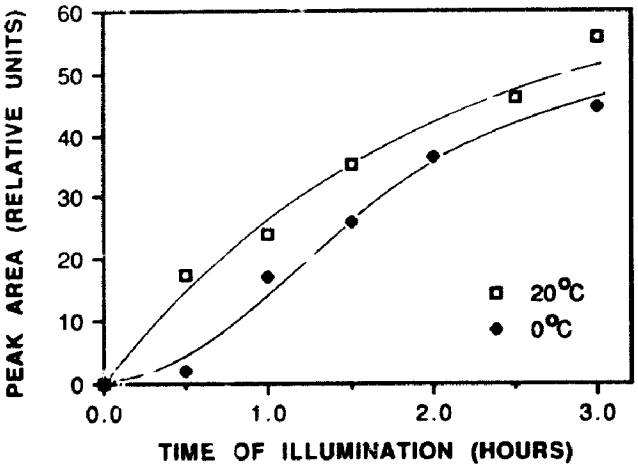

Fig. 4. Temperature dependence of the appearance of the 24 kDa breakdown fragment of D1. The data are derived from densitometer scans of an α-D1 western blot.

was dependent on the photochemical activity of the isolated reaction centre complex. Photochemical activity of the isolated complex can rapidly be inhibited by short periods of illumination in the absence of any electron donors or acceptors. In this figure it can be seen that when such pretreatment was used to decrease the reaction centre electron transfer activity by 50% (measured as the rate of electron flow from Mn^{2+} to silicomolybdate; (see Ref. 15)), the appearance of the 24 kDa product during subsequent illumination in the presence of DBMIB was significantly retarded. This result indicates that the generation of the 24 kDa fragment is dependent on reaction centres initially being photochemically active.

Fig. 4 shows that the degradation of the D1-polypeptide to the 24 kDa fragment during illumination in the presence of 200 μM DBMIB is temperature-sensitive during the initial 30 min of treatment. This result supports the findings of others working on larger particles, who found that the degradation of the D1-polypeptide in isolated thylakoids and PS II cores was significantly influenced by temperature over the same range [1,5]. We did observe, however, that with longer illumination times the effect of the two different temperatures became less obvious. We have also checked the pH optimum for the degradation process and found it to be about pH 8.0. As can be seen in Fig. 5, this is not the pH optimum for charge separation in the isolated complex as measured by DBMIB-dependent photoreduction of cytochrome *b*-559 (this being pH 7–7.5). Therefore, it seems that although the D1-polypeptide cleavage activity of the reaction centre complex is dependent on its photochemical activity, the proteolytic event can be partially separated from this activity based on pH optima. A similar pH optimum (pH 8.2) has recently been found for an intrinsic endopeptidase activity of the isolated PS II reaction cen-

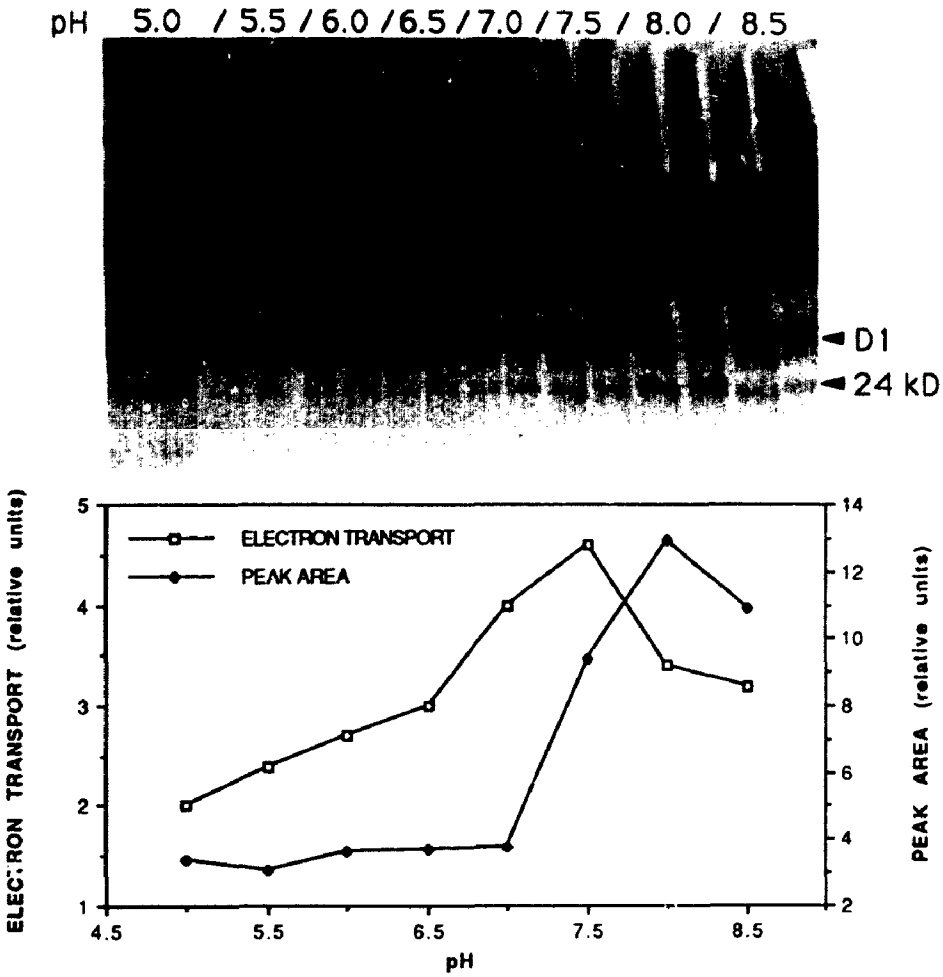

Fig. 5. The pH dependency of the breakdown of the D1-polypeptide to the 24 kDa fragment, and charge separation activity of the isolated PS II reaction centre complex is shown. At each pH value (between 5.0 and 8.5) illumination was performed for 30 and 45 min at 20 °C. These samples were analysed by SDS-PAGE and western blotting and the resulting blot was scanned for quantification of the 24 kDa breakdown product peak area (see graph). Each point on the graph is the average of the two blot lanes for each pH point. Charge separation activity of these samples was measured prior to illumination, using Mn^{2+} as an electron donor and DBMIB as an acceptor. The reduced quinone donates electrons to cytochrome *b*-559 and activity of the centres was measured as the rate of initial reduction of this cytochrome monitored at 559 nm.

tre detected using an exogenous peptide substrate [16]. This same study indicated that the activity could be reduced by inhibitors of serine-type proteinases. Using PS II cores, Virgin et al. [17] have also found that the photodegradation of the D1-polypeptide could be inhibited by serine-type proteinase inhibitors. For this reason we tested a range of proteinase inhibitors to see whether a similar inhibition of the appearance of the 24 kDa fragment would occur in the isolated PS II reaction centre. In agreement with previous findings, we found that significant inhibition of D1-polypeptide breakdown could be observed if proteinase inhibitors were present, with the most striking results being obtained with PMSF and soybean trypsin inhibitors (Table I).

Discussion

Our results indicate that when an effective electron acceptor is present, such as DBMIB, illumination of the isolated PS II reaction centre brings about a degradation of the D1-polypeptide yielding a characteristic breakdown pattern, dominated in our immunoblots by a fragment of about 24 kDa. No such pattern is observed in the absence of an acceptor. A 24 kDa fragment has also been detected in other experiments involving more complex in vitro systems [1,5] and may be the same D1-fragment as that observed in vivo (Refs. 3 and 4; Cook and Barber, unpublished results). The appearance of this fragment can occur under aerobic and anaerobic conditions and is unaffected by

TABLE I

Levels of inhibition of degradation of the D1-polypeptide, to a 24 kDa fragment, afforded by various proteinase inhibitors

All proteinase inhibitors were present in an equimolar concentration with the PS II reaction centre. The data presented here were calculated from densitometry of α-D1 western blots; each figure is an average of four scanned blot lanes.

Proteinase inhibitor	Class	Inhibition of production of the 24 kDa fragment (%)
Phenanthroline monohydrate	metallo	10
Soybean trypsin inhibitor	trypsin-like serine	~95
PMSF	serine and thiol	70
Antipain	trypsin-like serine and cysteine	30
TPCK	chymotrypsin-like serine	10
E-64	cysteine	0

the presence of a free radical scavenger. Proteolytic cleavage seems to be brought about by a serine-type endopeptidase activity [16,17] with a pH optimum at about 8.0. That the photoinduced loss of D1-polypeptide is inhibited at pH values below 7 has also been shown by Reisman and Ohad [18]. We conclude, as we did previously [8], that the endopeptidase activity is located within the isolated PS II reaction centre complex itself, indicating that one or more of its components (D1, D2, α and β subunits of cytochrome *b*-559 or product of the *psb*I gene) is able to proteolytically cleave the D1-polypeptide as a result of photochemical damage.

We have previously shown that in the presence of DBMIB, the action of strong illumination is to cause initially an oxidation and subsequent destruction of the β-carotenes bound to the isolated PS II reaction centre complex [19]. This irreversible loss of β-carotene is also accompanied by the degradation of chlorophylls, especially those species that absorb at 680 nm [20]. The loss of these pigments would be expected to bring about conformational changes that could trigger D1-degradation, perhaps allowing a serine residue to interact with a peptide linkage so as to facilitate the proteolytic cleavage. Where the active serine is located and how precisely the proteolytic events lead to D1-breakdown have yet to be elucidated. Clearly, further studies with the isolated reaction centre of PS II should yield new and valuable information to answer these key questions.

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