β-BROMO-β-NITROSTYRENE AS A FACILE AND PHOTOSYSTEM I-SPECIFIC ELECTRON ACCEPTOR

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1. Introduction

Determination of photosystem I activity in chloroplasts is predominantly carried out by measuring NADP reduction, which implies addition of ferredoxin to the isolated chloroplasts. With chloroplast fragments, obtained for example by digitonin treatment of chloroplasts [1], photosystem I activity is also measured as a rule by NADP reduction, which requires addition of plastocyanin, ferredoxin and ferredoxin-NADP reductase to the fragments. Another way of measuring photosystem I activity is to determine the oxygen uptake by addition of methyl viologen to the preparations. The latter method, however, requires an oxygraph.

In a previous paper we introduced β-bromo-β-nitrostyrene (BNS) as an energy transfer inhibitor in chloroplast photophosphorylation [2]. The compound showed an absorption maximum at 330 nm, which disappeared on reaction with SH-compounds [3]. Upon further examination the absorption at 330 nm of BNS also disappeared during illumination of chloroplasts or photosystem I particles, but not during illumination of chloroplasts in which plastocyanin was inactivated by cyanide treatment [4]. Thus BNS is obviously an electron acceptor specific for photosystem I. The phototransformation of BNS by chloroplasts or by photosystem I particles could be performed without addition of ferredoxin. The extinction coefficient for BNS at 330 nm is $10 \cdot 10^6$ cm^2/mol, which implies that the photoreductive capacity of photosystem I can be measured easily and sensitively.

This paper introduces BNS for the convenient spectroscopic determination of photosystem I activity.

Abbreviations used: DCIP, 2,6-dichlorophenolindophenol; DCMU, 3,4-dichloro-phenyl-1,1-dimethylurea; TES, *N*-tris (hydroxymethyl) methyl-2-aminoethanesulfonic acid; TMPD, tetramethyl-*p*-phenylene-diamine; tricine, *N*-tris (hydroxymethyl) methylglycine.

2. Materials and methods

Spinach chloroplasts were isolated by grinding leaves in 0.04 M TES (pH 7.3) and 0.35 M NaCl during 30 sec in an Omnimixer at 0°C. The homogenate was then filtered through cheese-cloth and chloroplasts were sedimented by 5 min centrifugation at 2000 × *g* at 0°C. The chloroplasts were washed once in 0.03 M tricine (pH 7.3), 0.2 M sucrose and 0.01 M NaCl and suspended in the same medium.

Stroma lamella photosystem I particles were prepared by 0.2% digitonin treatment of chloroplasts [1]. Inactivation of plastocyanin in chloroplasts was effected by cyanide treatment [4].

Decline in absorption at 330 nm was measured in a Cary R14 spectrophotometer at 20°C with side illumination as outlined before [3]. The scatter filter was UG 2 Schott. Ferricyanide reduction and NADP reduction were recorded as described elsewhere [3].

Oxygen uptake was determined in a Gilson oxygraph; illumination conditions were as in the Cary experiments. Oxygen concentration at 20°C was assumed to be 0.288 μmol/ml. BNS was dissolved in methanol and added as a methanolic solution; final concentration of methanol in reaction mixtures was 1% (v/v). In the dark BNS showed no reaction with any of the compounds used in experiments with BNS

described in this paper. Tris buffer must be avoided since BNS reacts slowly with this buffer. As mentioned, BNS reacts with SH-compounds, including ferredoxin and ferredoxin-NADP reductase [3].

3. Results and discussion

In fig.1 measurements of photochemical activities of chloroplasts are illustrated as the light-dependent decrease of BNS absorption at 330 nm owing to phototransformation of BNS. As stated earlier BNS also acts as an energy-transfer inhibitor. Therefore when water is the electron donor the addition of the uncoupler NH_4Cl is obligatory and with the ascorbate-DCIP couple as electron donor the uncoupler stimulates activity because of DCIP donating electrons partly after the coupling site and partly prior to this site. Maximum electron transport activity occurred at pH 7.8 and the highest activity was obtained with the ascorbate-DCIP couple in the presence of NH_4Cl, i.e., 77 μmol BNS reduced per hour per mg chlorophyll.

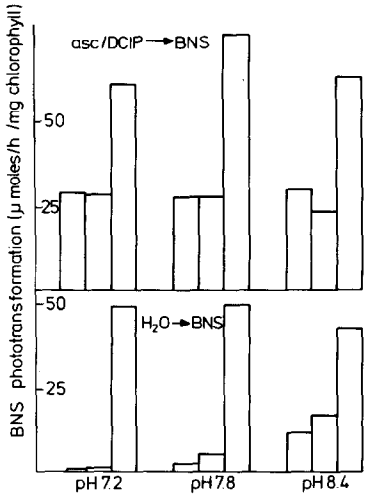

Fig.1. Photoreduction of BNS by chloroplasts. In all groups of blocks the left block represents activity without ADP and P_i, the middle block represents activity with ADP and P_i, and the right block represents activity with ADP, P_i and NH_4Cl added. The reaction medium (3.0 ml) contained in μmoles: tricine, 125; $MgSO_4$, 10; BNS, 0.3; and when added, ADP, 1; P_i, 10; NH_4Cl, 15. With ascorbate/DCIP as donor couple: ascorbate, 20; DCIP, 0.6; DCMU, 0.03. Chloroplasts containing 30 μg of chlorophyll were used.

Although this value seems rather low, it should be taken into account, as mentioned later in this paper, that BNS is a 4-electron acceptor. Therefore the 77 μmol BNS reduced implies an electron turn-over of 308 per hour per mg by photosystem I in the chloroplasts.

In the same system, but with NADP as the acceptor and nigericin as the uncoupler, Shavit and Shoshan [5] observed an NADP photoreduction amounting to 140 μmol/h/mg chlorophyll.

As NADP is a 2-electron acceptor, electron turn-over by photosystem I was 280. The value of 308 obtained with BNS is therefore quite acceptable.

As the results, using only photosystem I, were quite satisfactory it seemed interesting to trace whether reduction of BNS is performed by photosystem I only or whether there is a second reduction site between photosystems II and I. Ouitrakul and Izawa [4] reported inactivation of plastocyanin in chloroplasts by cyanide treatment of the chloroplasts. As BNS showed no reaction with oxidized or reduced plastocyanin [3] the possibility of a photoreduction of BNS before the plastocyanin site by using plastocyanin-deficient chloroplasts was examined.

As shown in table 1, in the control with ferricyanide some 90% inhibition was obtained with the cyanide treatment and with BNS over 90% inhibition was recorded.

These results suggest that BNS is an electron acceptor specific to photosystem I.

Wessels and Voorn [1] reported the preparation of subchloroplast vesicles containing only photosystem I by treatment of chloroplasts with low concentrations

Table 1
Photoreduction of ferricyanide and BNS by cyanide-treated chloroplasts

Acceptor	Treatment	Reduction μmol/h/mg chlorophyll
ferricyanide	none	467
ferricyanide	cyanide	52
BNS	none	50
BNS	cyanide	3

Reaction medium as in fig.1, but with NH_4Cl added throughout. Ferricyanide reduction was measured at the optimum pH 7.2 [6] and BNS reduction at the optimum pH 7.8.

Table 2
Photoreduction of BNS, methyl viologen and NADP by photosystem I particles

Electron donor couple	Acceptor	Activity μmol/h/mg chlorophyll
Ascorbate/DCIP	BNS	412 BNS reduced
Ascorbate/DCIP	BNS	430 O_2 uptake
Ascorbate/DCIP	NADP	110 NADP reduced
Ascorbate/DCIP	Methyl viologen	731 O_2 uptake
Ascorbate/TMPD	BNS	560 BNS reduced
Ascorbate/TMPD	BNS	573 O_2 uptake
Ascorbate/TMPD	Methyl viologen	1242 O_2 uptake
Ascorbate/TMPD	BNS + methyl viologen	955 O_2 uptake

Reaction medium as in fig.1 (the amount of TMPD added was the same as that of DCIP) with NH_4Cl added throughout and pH 7.8. In the oxygen uptake measurements 25 units catalase (Sigma C-100) and 0.17% (v/v) ethanol were added to avoid decomposition of H_2O_2 which, in any case with methyl viologen, was the final product of electron transport. Particles containing 5 μg chlorophyll were used in the BNS and methyl viologen experiments. With NADP 30 μg chlorophyll was used to obtain accurate determinations. With BNS and methyl viologen as acceptor 0.1 mg plastocyanin was added, which stimulated activities by about 10%. With NADP as acceptor, additions were as stated in ref. [1]. The oxygen uptake rates are listed after correction for oxygen uptake without BNS or methyl viologen; with ascorbate/DCIP this uptake was considerable (390 μmol O_2/h/mg chlorophyll), with ascorbate/TMPD it was small (90 μmol/h/mg chlorophyll).

of digitonin. Results obtained with these photosystem I particles are listed in table 2. In the first place it can be seen that the reduction rate of BNS greatly exceeds that of NADP. The oxygen uptake rate with the 2-electron acceptor methyl viologen, using the most effective donor couple ascorbate/TMPD, was 1242 μmol/h/mg chlorophyll, which implies a transfer of 2484 μM equivalents by photosystem I. Reduced BNS turned out to react stoichiometrically with oxygen (see below). With the 4-electron acceptor BNS the oxygen uptake rate was 573, which implies a transfer of 2292 μM equivalents by photosystem I. Thus with both acceptors the electron turn-over rate of photosystem I was almost equal. Therefore in measurements of the electron turn-over capacity of photosystem I methyl viologen and BNS are equally effective. With BNS plus methyl viologen added the oxygen uptake rate was the average of the separate determinations, and thus no preference for one of the acceptors was evident. With the best electron donor couple, i.e., ascorbate/TMPD, a rather flat pH optimum at 7.8 was recorded with BNS, as illustrated in fig.2. The equimolar reaction between reduced BNS and oxygen was concluded from measurements, using the ascorbate/TMPD couple in the oxygraph, and adding pulses of BNS. A calculation was then made of the total amount of extra oxygen taken up until the uptake rate returned to the level before addition of BNS. Addition of 50 nmol BNS resulted in an extra uptake of 46 nmol oxygen, and addition of 100 nmol BNS resulted in an extra uptake of 101 nmol oxygen. Thus we can safely

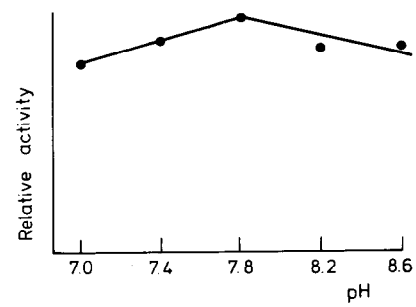

Fig.2. Photoreduction of BNS by photosystem I particles with ascorbate/TMPD as the donor couple. Reaction conditions as in table 2.

assume that reduced BNS reacts with oxygen in an 1 : 1 way. Knowing this it was possible to determine how many electrons are taken up by BNS by using water as the electron donor and measuring the net result concerning oxygen evolution at the watersplitting site and oxygen uptake at the BNS reduction site. In these determinations, with 0.3 μmol BNS plus 15 μmol NH_4Cl added, the net result was nil, and therefore we may assume that one mole of oxygen was taken up for every mole evolved. The latter implies splitting of four moles of water and the transport of four electrons to BNS. (With the same chloroplasts and 0.1 μmole methyl viologen in substitution for BNS the oxygen uptake rate was 173 μmol/h/mg chlorophyll.) Thus BNS is a very efficient, easily determinable and specific electron acceptor of photosystem I, accepting four electrons and reacting with one mole of oxygen. The reaction with oxygen does not result in the reappearance of the 330 nm band. The mechanism of the phototransformation of BNS is under further investigation.

References

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