ELSEVIER

Biochimica et Biophysica Acta 1187 (1994) 80–88

BBA Biochimica et Biophysica Acta

The role of tryptophan in the reaction catalyzed by spinach ferredoxin-dependent nitrite reductase

M. Hirasawa [a], P.A. Proske [b], D.B. Knaff [a,c,*]

[a] *Department of Chemistry and Biochemistry, Texas Tech University, Lubbock, TX 79409-1061, USA*
[b] *Howard Hughes Medical Institute, University of Texas Southwestern Medical Center, Dallas, TX 75235-9050, USA*
[c] *Institute for Biotechnology, Texas Tech University, Lubbock, TX 79409-1061, USA*

Received 14 March 1994

Abstract

Treatment of nitrite reductase with an 8–10-fold excess of *N*-bromosuccinimide (NBS) for 16–24 h modifies slightly less than 1 mol of tryptophan per mol of enzyme and eliminates approx. 80% of the activity of the enzyme, whether the physiological electron donor, reduced ferredoxin, or the non-physiological donor, reduced methyl viologen, is used as a source of electrons. NBS treatment does not result in any detectable change in the secondary structure of the enzyme and does not alter the oxidation-reduction properties of the siroheme and [4Fe-4S] cluster prosthetic groups of the enzyme. NBS has little or no effect on the affinity of substrate binding by nitrite reductase. These data suggest the possibility that one of the eight tryptophan residues known to be present in spinach nitrite reductase may play a direct role in the electron transfer reaction catalyzed by the enzyme. Data presented below suggest that this tryptophan may be proximal to both the ferredoxin-binding site and the siroheme group.

Key words: Nitrite reductase; Ferredoxin-dependent enzyme; Tryptophan; Electron transfer

1. Introduction

Spinach chloroplasts contain an enzyme, ferredoxin :nitrite oxidoreductase (EC 1.7.7.1), hereafter referred to as nitrite reductase) that catalyzes the 6-electron reduction of nitrite to ammonia using reduced ferredoxin as the electron donor [1]. The complete amino acid sequence of the monomeric 63 kDa spinach enzyme has been deduced from the base sequence of a cDNA clone [2] and the enzyme is known to contain a single siroheme ($E_m = -290$ mV) and a single [4Fe-4S] cluster ($E_m = -365$ mV) as prosthetic groups [1,3,4].

Nitrite reductase forms an electrostatically stabilized complex with its physiological electron donor, ferredoxin [1,5–9], that appears to involve negatively charged residues on ferredoxin [8,9] and positively charged residues on nitrite reductase [8,10]. These electrostatic interactions appear to play an important role in the docking of the proteins that is required for electron transfer from reduced ferredoxin to the enzyme [4]. While chemical modification techniques have proven useful in establishing the role of charged residues in the binding of ferredoxin to nitrite reductase [9,10], modification studies had not yet been used to investigate the possible role of specific amino acids in the electron transfer reaction per se. As it has been proposed that aromatic amino acid residues may facilitate electron transfer reactions in proteins (see Ref. [11] for a review and Refs. [12,13] for more recent examples), it seemed useful to investigate the effect of modifying aromatic amino acids in nitrite reductase. Below we present the results of a study on the effects of modification of nitrite reductase by *N*-bromosuc-

Abbreviations: CD, circular dichroism; DTNB, 5,5′-dithiobis-(2-nitrobenzoic acid); NBS, *N*-bromosuccinimide; NiR, nitrite reductase.

* Corresponding author. Fax: +1 (806) 742-1289.

SSDI 0005-2728(94)00059-E

cinimide (NBS), a reagent known to be selective for the modification of tryptophan [14–16], that suggest a possible role for at least one nitrite reductase tryptophan in electron transfer from reduced ferredoxin to the enzyme.

2. Methods

Ferredoxin ($A_{422nm}/A_{277nm} = 0.45$) and nitrite reductase ($A_{388nm}/A_{278nm} = 0.63$) were prepared as described previously [10] and stored at liquid nitrogen temperature in 30 mM Tris-HCl buffer (pH 8.0) and 250 mM potassium phosphate buffer (pH 7.7), respectively, until used. Ferredoxin concentrations were estimated using an extinction coefficient at 422 nm of 9.7 mM^{-1} cm^{-1} [17]. Nitrite reductase concentrations were measured by estimating the protein concentrations of nitrite reductase stock solutions using the method of Bradford [18], with bovine serum albumin as a standard. Antibody raised against nitrite reductase was prepared as described previously [19].

NBS, 5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB), chloramine T and molecular weight standards for gel filtration were purchased from Sigma. Ouchterlony immunodouble diffusion plates were purchased from Cooper. Isoelectric focusing standards (p*I* 2.80–6.55), Ultrogel AcA 44 and CNBr-activated Sepharose 4B were purchased from Pharmacia. Acrylamide (electrophoresis grade) and Western Blot kits were purchased from Bio-Rad.

Nitrite reductase activity was measured as described previously [20]. Co-migration of nitrite reductase/ferredoxin complexes during gel filtration chromatography was evaluated as described previously [9], except that enzyme activity rather than absorbance was used to monitor the presence of nitrite reductase. Ferredoxin affinity chromatography was carried out using ferredoxin coupled covalently to Sepharose 4B according to the procedure of Shin and Oshino [21]. Isoelectric focusing was performed using a Pharmacia Phast-system, according to directions supplied by the vendor. Western blots were performed as described previously [22]. Oxidation-reduction titrations were performed electrochemically using a gold mini-grid electrode and a Bioanalytical Systems Model CV-27 potentiostat, essentially as described previously [3,4]. Absorbance spectra were measured using a Shimadzu Model UV2100U spectrophotometer and circular dichroism (CD) spectra were measured using either a JASCO Model J-20 or Model 600 spectropolarimeter. All spectra were measured at ambient temperature. Amino acid analyses were performed using PICO-TAG™ chemistry and a Waters Model 840 chromatography and data analysis system. All amino acid analysis values were the average of three independent runs. A picomole ratio of NBS-treated nitrite reductase to native enzyme was calculated and these values were then normalized so that the value for valine was 100%.

NBS treatment of nitrite reductase was carried out by adding an aliquot of freshly prepared NBS stock solution to the enzyme sample (nitrite reductase concentrations ranging from 0.3 to 1.4 mM, in either 10 mM or 250 mM potassium phosphate buffer (pH 7.7), were used) and incubating the mixture in the dark at 4°C. After the indicated incubation time, the reaction mixture was diluted between 1000- and 20 000-fold with 250 mM potassium phosphate buffer (pH 7.7) and the diluted mixture centrifuged in an Amicon Microcentricon concentrator (50 kDa molecular mass cut-off) to stop further modification. The activity of the enzyme was then assayed, using both ferredoxin and methyl viologen as electron donors. The extent of modification of the eight tryptophan residues present on nitrite reductase [2] was determined using the method of Spande and Witkop [14]. The number of cysteines modified by NBS was calculated by determining the number of cysteines present, before and after NBS treatment, using the DTNB method of Janatova et al. [23].

Fluorescence excitation and emission spectra were measured at ambient temperature using either a Perkin Elmer Model MFP3 or a SLM Instruments Model 4800C spectrofluorometer at 2 nm spectral resolution. K_d values for the binding of ferredoxin to nitrite reductase were measured using a modification of the tryptophan quenching procedure of Davis [24], developed for measuring ferredoxin binding to ferredoxin: $NADP^+$ oxidoreductase. Ferredoxin fluorescence was virtually negligible at concentrations between 1 μM and 10 μM [24]. In experiments in which ferredoxin concentrations > 10 μM were used, the component of the total fluorescence arising from ferredoxin was subtracted in order to measure the fluorescence of nitrite reductase. The K_d for nitrite binding to the enzyme was measured from the quenching of nitrite reductase fluorescence by nitrite in a similar fashion and also from plots of the spectral perturbation caused by nitrite binding to the enzyme, ($\Delta A_{388-408nm}$) vs. [NO_2^-], as described previously [25]. In experiments in which acrylamide was used as a fluorescence quencher, a correction for the absorbance of acrylamide at 295 nm was made [26].

3. Results

Fig. 1 shows the effect of treating nitrite reductase with the tryptophan-modifying reagent, NBS, on the activity of the enzyme. Activities with the physiological electron donor, reduced ferredoxin, and with the non-physiological donor, reduced methyl viologen, were

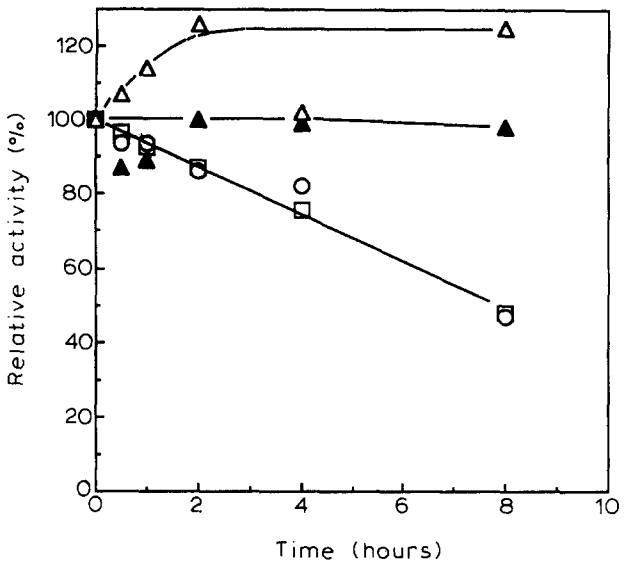

Fig. 1. The effect of NBS treatment on the activity of spinach nitrite reductase. Nitrite reductase was treated with a 8-fold excess of NBS in 250 mM potassium phosphate buffer (pH 7.7) and aliquots removed for activity assays at the indicated times. 100% specific activity corresponds to 135 units mg^{-1} for methyl viologen-linked and 188 units mg^{-1} for ferredoxin-linked activities, respectively. The open squares and circles represent ferredoxin-linked and methyl viologen-linked activities, respectively. The closed and open triangles represent, respectively, the ferredoxin-linked and methyl viologen-linked activities of a nitrite reductase sample that was incubated with an 8-fold excess of NBS as a 1:1 mixture of enzyme plus ferredoxin in 10 mM potassium phosphate buffer for the indicated times.

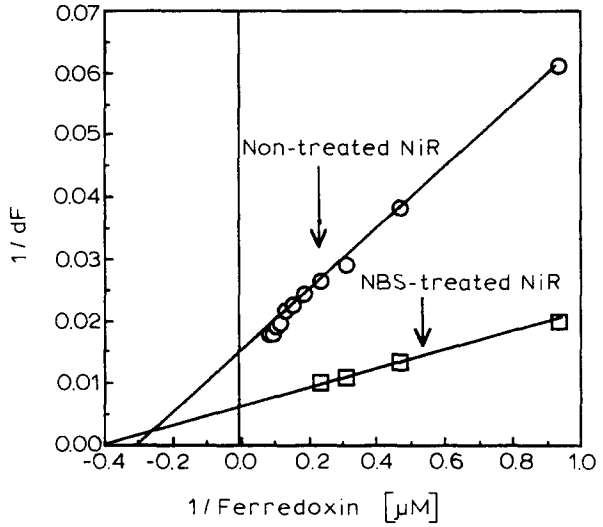

Fig. 2. The effect of ferredoxin concentration on the tryptophan fluorescence of nitrite reductase. The fluorescence intensity at 330 nm of a 5 μM solution of nitrite reductase in 10 mM potassium phosphate buffer (pH 7.7) was measured using 295 nm excitation.

affected to the same extent. These results are in contrast to those obtained previously with the lysine modifying-reagent *N*-acetylsuccinimide, and the arginine-modifying reagent phenylglyoxal, both of which inhibit the ferredoxin-linked activity of nitrite reductase without affecting the methyl viologen-linked activity [10]. Although it was not possible to obtain complete inhibition of enzyme activity by treatment with 8–10-fold molar excesses of NBS, longer incubation times resulted in inhibitions close to 80% (see below). The pattern observed in Fig. 1, with both the ferredoxin-linked and methyl viologen-linked activities inhibited to the same extent, was also observed at longer incubation times (see below).

Table 1 summarizes the effects of treating nitrite reductase with NBS for a longer period of time, i.e., 24 h, on the kinetic parameters of the enzyme and on the binding affinity of the enzyme for ferredoxin and for nitrite. Neither the K_d for the binding of ferredoxin (Fig. 2) nor the K_m for ferredoxin (Fig. 3) is appreciably affected by NBS treatment. The NBS-treated enzyme retained the ability of the native enzyme [1] to bind to a ferredoxin-Sepharose 4B affinity column and to co-migrate with ferredoxin during gel filtration chromatography at low ionic strength (data not shown), providing additional evidence for the conclusion that NBS treatment of nitrite reductase did not lessen the ability of the enzyme to form an electrostatically stabilized complex with ferredoxin.

It proved somewhat more difficult to compare the nitrite-binding capacities of the native and NBS-treated enzymes. Plots of rate vs. nitrite concentration were sigmoidal for both the native enzyme [25] and the NBS-treated enzyme (data not shown), with very similar half-maximal nitrite concentrations (Table 1). Both the fluorescence quenching and spectral perturbation assays described in Methods gave one-component binding isotherms with K_d values of 5 and 10 μM, respectively, for nitrite binding by the NBS-treated enzyme (Table 1). These values, which agree with one another to within the experimental uncertainties of the measurements, are also identical (to within the experimental uncertainties) with the K_d value of 10.3 μM re-

Table 1
Kinetic and substrate-binding properties of nitrite reductase

Substrates	V_{max} (%) [a]		Apparent K_m (μM)		K_d (μM)	
	native	+NBS	native	+NBS	native	+NBS
Ferredoxin	100	20	40	40	3.0 [b]	2.5 [b], 5.0 [c]
Nitrite	100	20	400 [d]	500 [d]	2.5 [b]	2.5 [b], 10 [e]

Nitrite reductase was treated with an 8-fold molar excess of NBS at 4°C for 24 h in 250 mM potassium phosphate buffer (pH 7.7). All activities were measured using reduced ferredoxin as the electron donor. [a] 100% activity corresponds to 14300 units. K_d values were measured either by fluorescence quenching [b] or by spectral perturbation ($\Delta A_{575-605nm}$ [c], $\Delta A_{388-408nm}$ [e]), respectively. In the case of sigmoidal binding curves, concentrations at which half-maximal fluorescence quenching was observed were used. [d] Nitrite concentrations that produced half-maximal rates were used.

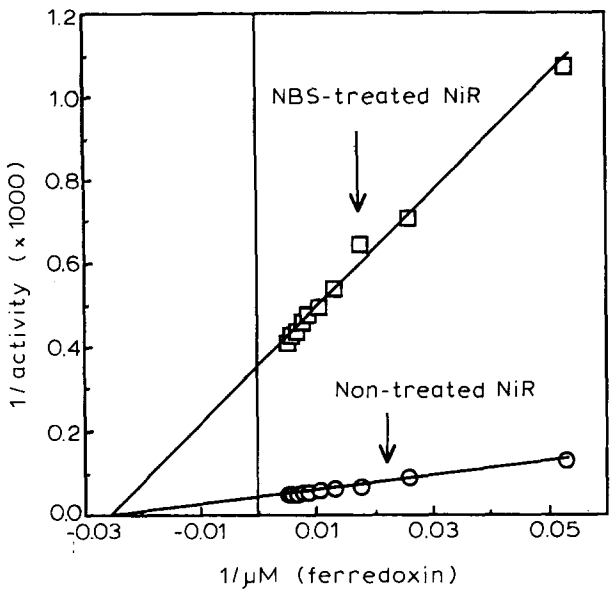

Fig. 3. The effect of ferredoxin concentration on the initial velocities of nitrite reduction catalyzed by native and NBS-treated nitrite reductase. Enzyme activity was measured as described in Methods, except that the ferredoxin concentration was varied as indicated.

ported for the native spinach enzyme by Mikami and Ida [6]. These K_d values are also virtually identical to the half-maximal nitrite concentration values measured in our laboratory from tryptophan fluorescence quenching titrations (Table 1) and from the titration of spectral perturbations [25] observed when nitrite is added to the native spinach enzyme. However, unlike the enzyme preparation used by Mikami and Ida [6], in our hands the nitrite-binding curves for native spinach nitrite reductase are sigmoidal and the data cannot be fitted to a single binding isotherm [25]. It appears likely that the sigmoidal nature of these binding curves arises from heterogeneity in preparations of nitrite reductase that arise, in turn, from proteolysis near the amino terminus of the enzyme during isolation and purification [2,3]. It is not completely clear why NBS treatment of the enzyme would eliminate heterogeneity in nitrite binding (see Discussion), but this phenomenon complicates analysis of the effect of NBS treatment on nitrite binding. Nevertheless, it can be said that NBS treatment does not eliminate the capacity of the enzyme to bind nitrite with good affinity.

In contrast to the minimal effects of NBS treatment on substrate binding and on the apparent K_m values for the two substrates, NBS treatment decreases the turnover numbers for both the ferredoxin-linked and methyl viologen-linked activities of nitrite reductase to 20% of the values observed with the native enzyme (Table 1, Fig. 3). The observation that NBS treatment inhibits activity with ferredoxin, the physiological electron donor, and with a non-physiological donor equally and the fact that NBS treatment has little effect on substrate binding (Table 1, Fig. 2), raise the possibility that the inhibitory effect of NBS treatment on enzyme activity may arise from modification of one or more amino acids directly involved in the electron transfer process. Before this possibility could be explored further, it was first necessary to eliminate the possibilities that the inhibition resulted from indirect effects such as NBS-induced conformational changes or alterations in the redox properties of the enzyme's prosthetic groups.

Western blots and Ouchterlony immunodouble diffusion experiments demonstrated that an antibody raised against native nitrite reductase recognized a sample of the enzyme that had been treated with a 10-fold molar excess of NBS for 24 h (data not shown), indicating that no conformational changes large enough to significantly affect the major antigenic epitopes accompany the loss of activity that results from NBS treatment. The CD spectrum of this NBS-treated nitrite reductase sample in the region from 190 to 230 nm (data not shown) was identical to that of the native enzyme [10], indicating that treatment of the enzyme with a 10-fold excess of NBS for 24 h did not produce any large changes in the secondary structure of the enzyme that could be responsible for the observed loss of activity. The CD spectra of the native and NBS-modified enzymes were also essentially identical between 350 and 750 nm (data not shown), indicating that the conformations of the siroheme-binding pocket and [4Fe-4S] cluster-binding region were not significantly altered by treatment with NBS. The pI of the NBS-treated enzyme, 4.2, is identical to that of the native enzyme, indicating that the loss of activity caused by NBS treatment does not arise from any change in the net charge on nitrite reductase caused by this tryptophan-modifying reagent. Oxidation reduction titrations of the siroheme and [4Fe-4S] prosthetic groups of a sample of nitrite reductase that had been treated with a 10-fold excess of NBS for 20 h gave E_m values of -290 mV ($n = 0.9$) and -375 mV ($n = 1.1$), respectively (data not shown). These values are identical, to within the experimental uncertainties, to those measured previously for the native enzyme [4].

Preincubation of nitrite reductase with an equimolar amount of ferredoxin at low ionic strength, conditions known to favor formation of an enzyme/ferredoxin complex [5], completely protected the enzyme against the loss of ferredoxin-linked and methyl viologen-linked activity caused by treatment with NBS. This is demonstrated in Fig. 1 for short-time incubation of the enzyme with NBS and is also true for longer incubation times. For example, the enzyme retained 98% of its ferredoxin-linked activity after 16 h of incubation of the ferredoxin/enzyme complex with an 8-fold molar excess of NBS, while treatment of nitrite reductase alone with NBS for the same amount of time resulted

in a loss of 80% of both the ferredoxin-linked and methyl viologen-linked activities. Somewhat surprisingly, treatment of the ferredoxin/nitrite reductase complex with NBS actually resulted in a small enhancement (approx. 25%) of the methyl viologen-linked activity of the enzyme (Fig. 1). Neither addition of ferredoxin at high ionic strength, where the enzyme/ferredoxin complex dissociates [5], nor addition of nitrite at either low or high ionic strength gave any protection against the loss of activity resulting from NBS treatment. The magnitude of the activity loss resulting from the incubation of nitrite reductase alone with NBS was the same in low and high ionic strength buffers (10 and 250 mM potassium phosphate, respectively), indicating that the protection observed in the presence of ferredoxin at low ionic strength arises from complex formation with ferredoxin rather than from a less effective reaction of the enzyme itself with NBS at low ionic strength. These results suggest that at least one amino acid located at or near the ferredoxin-binding site of nitrite reductase is involved in the electron transfer reaction catalyzed by the enzyme.

Analysis of the effect of NBS treatment on the tryptophan content of the enzyme suggests that the loss of activity described above is likely to be associated with the modification of a single tryptophan. Thus incubation of the enzyme with an 8-fold excess of NBS for 8 h, a treatment that produced a 50% loss in activity (Fig. 1), resulted in the loss of 0.54 tryptophan (determined as described in Ref. [14]) and incubation of the enzyme with NBS for 16 h, a treatment that produced a 80% loss in activity, resulted in the loss of 0.74 tryptophan. Detailed analyses of the tryptophan content of nitrite reductase peptides will be required to determine whether the total of slightly less than one tryptophan modified per molecule of enzyme under these conditions represents essentially complete modification of a single tryptophan or whether tryptophans at several loci are partially modified.

The observation that, despite the presence of eight tryptophans in spinach nitrite reductase, only approx. 1 mol of tryptophan per mol of enzyme was modified by NBS under the conditions described above suggested that the majority of tryptophan residues were either partially or completely buried and thus not exposed to the modifying reagent. Treatment of nitrite reductase with a 100-fold excess of NBS resulted in a modification of only 2.7 tryptophan residues, even after prolonged incubation of the enzyme with the modifying reagent (data not shown), suggesting that only three of the tryptophan residues in the enzyme are accessible to NBS. It appears likely that the remaining five tryptophans in the enzyme are buried in the interior of the protein and are thus inaccessible to the solvent. There must also be differences in the degree of solvent exposure of the three tryptophans that can be modified by NBS, as one is modified in the presence of an 8-fold molar excess of NBS, while the other two are modified only in the presence of much higher NBS concentrations.

Although NBS has been reported to be reasonably specific for the modification of tryptophan, it has been reported to also modify histidine, methionine, tyrosine and cysteine under some conditions [14–16]. Amino acid analysis of both native and NBS-treated nitrite reductase samples indicated that treatment of the en-

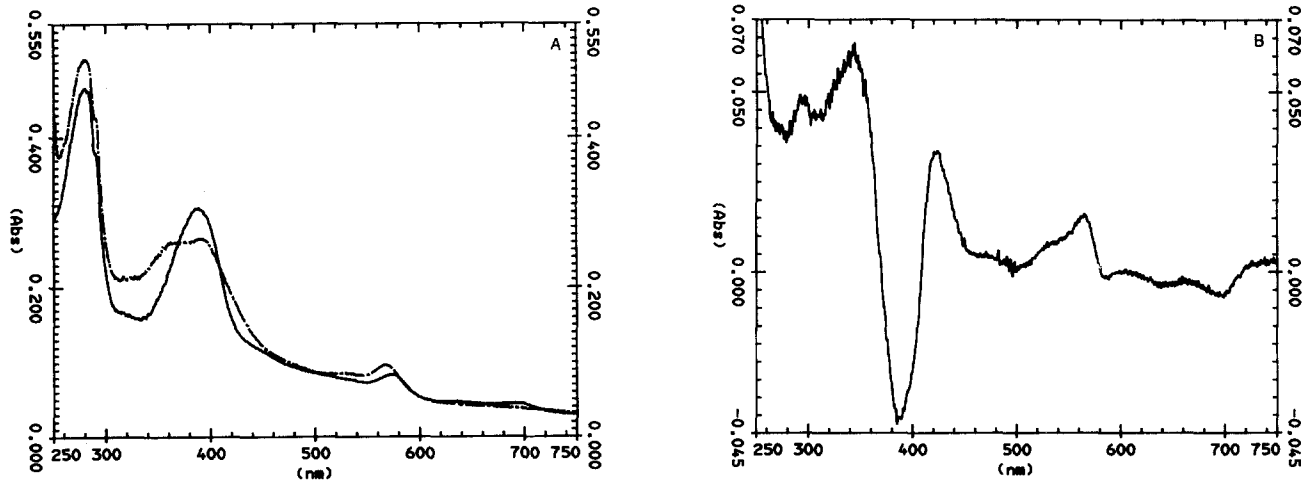

Fig. 4. Spectra of NBS-treated nitrite reductase and of its nitrite adduct. (A) The solid line represents the spectrum of a reaction mixture that contained, in 250 mM potassium phosphate buffer (pH 7.7), 2.9 μM nitrite reductase that had been treated with a 10-fold excess of NBS for 8 h in the same buffer and then diluted and centrifuged in an Amicon Centricon concentrator (30 kDa molecular mass cut-off) to remove excess NBS. The spectrum was obtained vs. a buffer blank in a 1.0 cm optical pathlength cuvette. The dashed line represents the spectrum of the same sample to which 100 μM sodium nitrite had been added. (B) The (NO_2^-· enzyme) minus enzyme difference spectrum of NBS-treated nitrite reductase. The NBS-treated nitrite reductase used in (A) was placed in both the sample and reference cuvettes and a baseline spectrum obtained. The difference spectrum was then generated by adding 100 μM sodium nitrite to the sample cuvette.

zyme with an 8-fold molar excess of NBS for 24 h had no effect, within the experimental uncertainties of the measurement, on the content of histidine, tyrosine or methionine (91%, 103% and 117% of the values found in the native enzyme were found in the NBS-treated enzyme for these three amino acids, respectively). Furthermore, treatment of the enzyme with a 16-fold molar excess of chloramine T, a reagent known to modify methionine residues [27,28], had no effect on the activity of the enzyme. Further evidence for the specificity of the NBS treatment was obtained from additional amino acid analysis data which showed that the treatment had no significant effect on the levels of glutamate plus glutamine, serine, glycine, arginine, threonine, alanine, proline, isoleucine, leucine, phenylalanine and lysine.

Treatment of nitrite reductase with an 8-fold molar excess of NBS for 24 h did decrease the cysteine content of the enzyme by 0.39 mol cysteine/mol enzyme. However, this loss of cysteine occurred almost immediately after the incubation period commenced (i.e., within 20 min after the addition of NBS) and no further loss of cysteine was observed. Thus, unlike the case for the loss of tryptophan described above, the time course for the modification of cysteine caused by NBS treatment did not parallel the time course for the loss of activity. Complex formation with ferredoxin, a treatment that completely protected nitrite reductase from the NBS-induced loss of activity (Fig. 1), also completely eliminated the loss of tryptophan caused by the addition of NBS (data not shown). In contrast, complex formation with ferredoxin had no effect on the loss of nitrite reductase cysteine caused by NBS. These results make it appear unlikely that the loss of activity caused by treating nitrite reductase with NBS arises from the modification of a small amount (i.e., less than one residue) of cysteine.

The absorbance spectrum of nitrite reductase treated with a 10-fold molar excess of NBS for 22 h at 4°C (Fig. 4A) is virtually identical to that of the native enzyme in the visible region (see Refs. [29,30]). A small decrease in absorbance in the ultraviolet is observed, as a result of the modification of approx. 0.8 tryptophan by NBS (see above). The absorbance spectrum of the nitrite adduct of the NBS-treated enzyme (Fig. 4A) differs slightly from that of the nitrite adduct formed by the native enzyme [29,30], with the Soret band maximum shifted from 396 nm in the native enzyme to 392.5 nm in the treated enzyme. In addition, a shoulder observed at 364 nm in the spectrum of the nitrite adduct of the NBS-treated enzyme (Fig. 4A), is not seen with the native enzyme [29,30]. Additional changes resulting from NBS treatment can be seen more clearly by comparing the difference spectrum of the nitrite adduct of the NBS-treated enzyme minus the spectrum of the NBS-treated enzyme in the absence of the ligand (Fig. 4B) to that of the native enzyme [25,29,30]. As nitrite is known to bind to the siroheme group of the enzyme [1], the observation that shifts in the spectrum of the nitrite adduct of nitrite reductase are caused by NBS modification of a tryptophan suggests that this tryptophan may be in the vicinity of the siroheme group.

Modification of a tryptophan residue(s) would be expected to lead to a change in the fluorescence properties of the enzyme. Fig. 5A and B show the fluorescence emission spectra of both native and NBS-treated nitrite reductase. (The native and NBS-treated enzymes had essentially identical excitation spectra, with a maximum at 295 nm.) The multiple emission maxima, in the region where tryptophan is known to fluoresce, observed in both emission spectra suggest a heterogeneity in the environments of the emitting tryptophans, both in the native and NBS-treated enzymes. Although there are some small differences in the shape

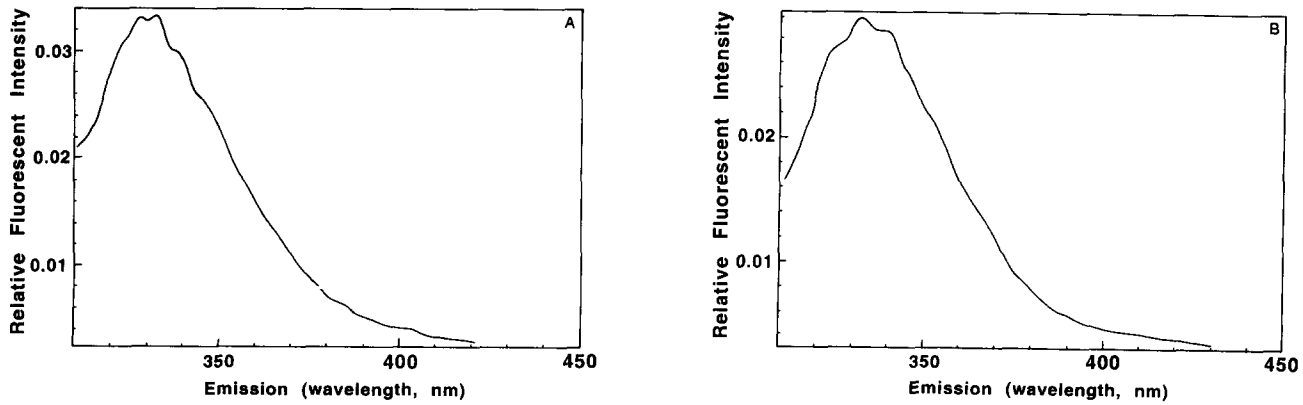

Fig. 5. Fluorescence emission spectrum of nitrite reductase. The reaction mixtures contained, in 250 mM potassium phosphate buffer (pH 7.7), either 2.4 μM native nitrite reductase (A) or 22.4 μM enzyme that had been treated with an 8-fold excess of NBS for 16 h (B). The excitation wavelength was 295 nm and both emission and excitation slit widths were set at 2 nm.

of the two emission spectra, the most striking effect of NBS treatment is the 90% decrease in tryptophan fluorescence yield. (Note that the concentration of NBS-treated enzyme used for the experiment of Fig. 5B is 9.3-fold higher than that of the native enzyme used to obtain the spectrum shown in Fig. 5A.) As NBS treatment of nitrite reductase modifies neither tyrosine nor phenylalanine residues on the enzyme (see above), the decreased fluorescence of the enzyme must result from a decrease in the fluorescence of one or more enzyme tryptophans. If NBS treatment modifies only a single tryptophan residue (see above), then this large decrease in the tryptophan fluorescence yield of nitrite reductase that accompanies NBS treatment implies that this tryptophan is characterized by a significantly greater fluorescence quantum yield than are the other seven tryptophans present in the enzyme.

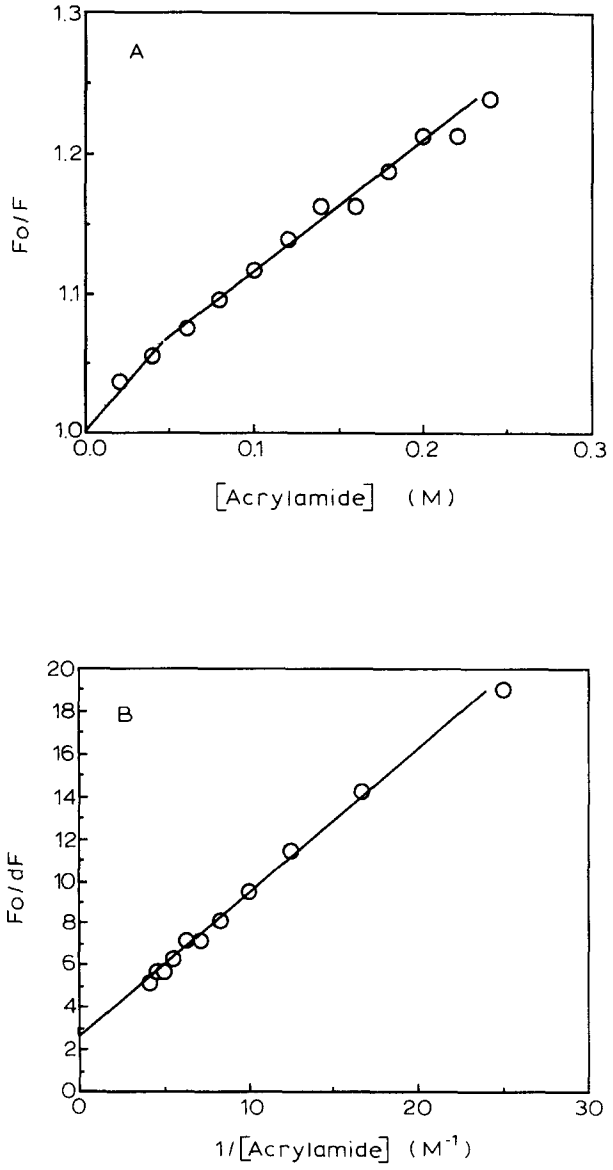

Fig. 6. The effect of acrylamide on the tryptophan fluorescence of nitrite reductase. The reaction mixture contained 5.6 μM nitrite reductase in 10 mM potassium phosphate buffer (pH 7.7). (A) Stern-Volmer plot. (B) Stern-Volmer plot modified according to Lehrer [31]. F_0 is the fluorescence intensity in the absence of acrylamide, F is the fluorescence in the presence of the indicated concentration of acrylamide, $dF = F - F_0$.

Additional evidence for heterogeneity in tryptophan environment in nitrite reductase was obtained from the results of acrylamide fluorescence quenching experiments. Fig. 6A shows that a Stern-Volmer plot for the quenching of nitrite reductase tryptophan fluorescence by the neutral quencher acrylamide [31] is non-linear. Analysis of the acrylamide quenching data according to the method of Lehrer [31] suggests that 37% of the tryptophans in nitrite reductase that contribute to the fluorescence are accessible to acrylamide and thus are likely to be exposed to the solvent (Fig. 6B). As we have no independent method for determining how many of the tryptophans do contribute significantly to the fluorescence of the enzyme, it was not possible to estimate the actual number of tryptophan residues that are accessible to acrylamide. It should be pointed out that some caution must be exercised in interpreting the non-linear Stern-Volmer plots observed for acrylamide fluorescence quenching, as exposure to acrylamide could perhaps cause conformational changes in the protein and has been reported to cause a small loss in the activity of ferredoxin : $NADP^+$ oxidoreductase [24]. In fact, exposure of nitrite reductase to 250 mM acrylamide inhibits both the ferredoxin-linked and methyl viologen-linked activities of the enzyme by 45%. However, the non-linearity in the Stern-Volmer plot (Fig. 6A) occurs at lower acrylamide concentrations (i.e., near 50 mM) where acrylamide does not inhibit the enzyme. (No inhibition of either ferredoxin-dependent or methyl viologen-dependent activity was observed even at an acrylamide concentration of 100 mM.)

4. Discussion

The data presented above demonstrate that modification of one tryptophan per nitrite reductase by NBS causes almost complete loss of enzymatic activity. It is not yet known whether a single specific tryptophan is completely modified by NBS treatment or whether partial modification of several different tryptophans occurs. However, the data suggest that modification of tryptophan in nitrite reductase by an 8–10-fold excess of NBS can occur at no more than three loci, as only three of the tryptophans present in the enzyme appear to be accessible to the tryptophan-modifying reagent. The additional observation that complex formation between nitrite reductase and ferredoxin completely eliminates both the loss of activity and the modification of tryptophan by NBS is consistent with the idea that only

a single tryptophan near the ferredoxin-binding site of the enzyme is modified by NBS under the conditions used in this study. The effects of tryptophan modification by NBS on the absorbance spectrum of the enzyme/nitrite adduct suggest that the modified tryptophan may also be in reasonable proximity to the siroheme prosthetic group of the enzyme.

Although the observation that NBS inhibition of the enzyme can be completely eliminated by pre-forming a complex of the enzyme with ferredoxin suggests that a tryptophan at or near the ferredoxin-binding site is altered by NBS, NBS treatment does not affect the binding of ferredoxin to the enzyme. While NBS treatment of the enzyme appears to have little effect on the ability of the enzyme to bind nitrite with good affinity, NBS treatment does eliminate the sigmoidicity seen in nitrite-binding curves obtained with the native enzyme (see above). As nitrite reductase contains a single siroheme and thus a single nitrite-binding site, the heterogeneity observed in nitrite binding by some, but not all, nitrite reductase preparations [6,25] is likely to arise from the known protein heterogeneity that can result from proteolytic cleavage of the enzyme during isolation and purification [2,3]. It appears that the true amino terminus of the mature form of the spinach enzyme is a cysteine [2], but that between 45% [3] and 75% [2] of the protein loses this cysteine due to proteolysis during purification. It may be possible that elimination of the free sulfhydryl group at the amino terminus, by NBS treatment, of that portion of the nitrite reductase molecules present in our preparation that has not undergone proteolytic treatment produces a preparation in which all nitrite reductase molecules have very similar affinities for nitrite. The fact that approx. 0.4 cysteine per enzyme is modified by NBS (see above) is at least consistent with this interpretation. However, it is not clear why modification of the amino terminal cysteine by NBS does not then eliminate the sigmoidal shape of the activity vs. [nitrite] profile (see above). Clearly additional mechanistic details that would allow an elucidation of the relationship between the apparent K_m and K_d are required before this question can be addressed further.

As reported above, NBS treatment of nitrite reductase appears to have no major effect on the secondary structure of the enzyme. Furthermore, NBS treatment has no effect on the E_m value of either the [4Fe-4S] cluster or siroheme found in the enzyme.

If the inhibition of nitrite reductase that results from the modification of one tryptophan by NBS cannot be attributed to alterations in secondary structure, substrate-binding or the redox properties of the prosthetic groups, the most likely remaining possibility is that this tryptophan is directly involved in the electron transfer reaction itself. If this is indeed the case, one would expect this tryptophan residue to be highly conserved in ferredoxin-dependent nitrite reductases. In fact, all eight of the tryptophans present in spinach nitrite reductase [2] are conserved in the three other higher plant, ferredoxin-dependent nitrite reductases for which sequences are available – maize [32], tobacco [33] and birch [34]. One of these, W34 (ignoring the transit peptide and designating the N-terminal cysteine of the spinach enzyme as amino acid 1), is located at a position that corresponds to the N-terminal methionine in the homologous ferredoxin-dependent nitrite reductase from the cyanobacterium Synechococcus sp. PCC 7942 [35,36]. A second tryptophan found in the spinach enzyme, W148, is located at a position occupied by the non-aromatic amino acid, leucine, in the Synechococcus enzyme [35,36]. If, as our chemical modification studies suggest, at least one tryptophan, because of its aromatic nature, is essential for the electron transfer activity of nitrite reductase, it thus appears unlikely to be either W34 or W148. Of the remaining six tryptophans found in spinach nitrite reductase, five are located at positions also occupied by tryptophan in the Synechococcus enzyme [35,36] and the sixth, W227, is located at a position occupied by the aromatic amino acid, phenylalanine, in the Synechococcus enzyme [35,36].

It should be pointed out that sulfite reductases found in non-photosynthetic bacteria, enzymes that catalyze the 6-electron reduction of sulfite to sulfide, contain the same siroheme and [4Fe-4S] cluster prosthetic groups and display significant sequence homologies to ferredoxin-dependent nitrite reductases found in photosynthetic organisms (see discussion in Ref. [35]). However, the bacterial enzymes utilize reduced pyridine nucleotide as the electron donor [37] and thus an aromatic amino acid involved in electron transfer from reduced ferredoxin to nitrite reductase in a photosynthetic organism might not be conserved in a bacterial enzyme. Nevertheless, of the six tryptophans found in spinach nitrite reductase that appear to be possible candidates for direct involvement in electron transfer reactions, three are found at positions (W227, W281 and W314) that are occupied by an aromatic amino acid – phenylalanine in one case and tyrosine in two cases – in the sulfite reductases from *Escherichia coli* and *Salmonella typhimurium* [35]. Definitive identification of the role of these aromatic amino acids will require future site-directed mutagenesis studies.

Acknowledgements

The authors would like to thank Mr. Jappe de Best, Ms. Michelle Dose and Ms. Hong Qin for their assistance in the initial stages of the isolation of nitrite reductase and ferredoxin, Dr. Yavuz Onganer for his assistance with the fluorescence measurements, Mr. B.

Fan and Prof. Y. Zhou for assistance with the CD measurements, Dr. Osamu Koguchi-Kamioka, Prof. R.W. Shaw, Prof. M.R. Ondrias and Prof. M.K. Johnson for helpful discussions and Ms. Carolyn Moomaw for advice on the amino acid composition measurements. This research was funded by a grant from the US Department of Energy (93ER20125.000 to D.B.K.) and by a donation from the Taka Saitoh Fund to M.H.

References

[1] Knaff, D.B. and Hirasawa, M. (1991) Biochim. Biophys. Acta 1056, 93–125.
[2] Back, E., Burkhart, W., Moyer, M., Privalle, L. and Rothstein, S. (1988) Mol. Gen. Genet. 212, 20–26.
[3] Hilliard, N.P., Hirasawa, M., Knaff, D.B. and Shaw, R.W. (1991) Arch. Biochem. Biophys. 291, 195–199.
[4] Hirasawa, M., Tollin, G., Salamon, Z. and Knaff, D.B. (1994) Biochim. Biophys. Acta 1185, 336–345.
[5] Hirasawa, M. and Knaff, D.B. (1985) Biochim. Biophys. Acta 830, 173–180.
[6] Mikami, B. and Ida, S. (1989) J. Biochem. 105, 47–50.
[7] Hirasawa, M., Gray, K.A., Sung, J.-D. and Knaff, D.B. (1989) Arch. Biochem. Biophys. 275, 1–10.
[8] Privalle, L.S., Privalle, C.T., Leonardy, N.J. amd Kamin, H. (1985) J. Biol. Chem. 260, 117–122.
[9] Hirasawa, M., Boyer, J.M., Gray, K.A., Davis, D.J. and Knaff, D.B. (1986) Biochim. Biophys. Acta 851, 23–28.
[10] Hirasawa, M., De Best, J.H. and Knaff, D.B. (1993) Biochim. Biophys. Acta 1140, 304–312.
[11] McLendon, G. and Hake, R. (1992) Chem. Rev. 92, 481–490.
[12] Farchaus, J.W., Wachtveitl, J., Mathis, P. and Oesterhelt, D. (1993) Biochemistry 32, 10885–10893.
[13] Hurley, J.K., Cheng, H., Xia, B., Markley, J.L., Medina, M., Gómez-Moreno, C. and Tollin, G. (1994) J. Am. Chem. Soc. 115, 11698–11701.
[14] Spande, T.F. and Witkop, B. (1967) in Methods in Enzymology (Hirs, C.H.W., ed.), Vol. 11, pp. 498–506, Academic Press, New York.
[15] Patchornik, A., Lawson, W.B. and Witkop, B. (1958) J. Am. Chem. Soc. 870, 4747–4748.
[16] Hasumi, H. and Nakamura, S. (1978) J. Biochem. 84, 707–717.
[17] Tagawa, K. and Arnon, D.I. (1969) Biochim. Biophys. Acta 153, 602–613.
[18] Bradford, M.M. (1976) Anal. Biochem. 72, 248–259.
[19] Hirasawa, M., Fukushima, K., Tamura, G. and Knaff, D.B. (1984) Biochim. Biophys. Acta 791, 145–154.
[20] Hirasawa, M. and Tamura, G. (1981) Agric. Biol. Chem. 45, 1615–1620.
[21] Shin, M. and Oshino, R. (1978) J. Biochem. 83, 357–361.
[22] Hirasawa, M., Sung, J.-D., Malkin, R., Zilber, A., Droux, M. and Knaff, D.B. (1988) Biochim. Biophys. Acta 934, 169–176.
[23] Janatova, J., Fuller, J.K. and Hunter, M.J. (1968) J. Biol. Chem. 3612–3622.
[24] Davis, D.J. (1990) Arch. Biochem. Biophys. 276, 1–5.
[25] Hirasawa, M., Shaw, R.W., Palmer, G. and Knaff, D.B. (1987) J. Biol. Chem. 262, 12428–12433.
[26] McClure, W.O. and Edelman, G.M. (1967) Biochemistry 6, 559–566.
[27] Sakurai, J. and Nagahara, M. (1985) Biochem. Biophys. Res. Commun. 128, 760–766.
[28] Schechter, Y., Burstein, Y. and Patchornik, A. (1975) Biochemistry 14, 4497–4503.
[29] Vega, J.M. and Kamin, H. (1977) J. Biol. Chem. 252, 896–919.
[30] Hirasawa, M. and Tamura, G. (1980) Agric. Biol. Chem. 44, 749–758.
[31] Lehrer, S.S. (1971) Biochemistry 10, 3254–3263.
[32] Lahners, K., Kramer, V., Back, E., Privalle, L. and Rothstein, S. (1988) Plant Physiol. 88, 741–746.
[33] Kronenberger, J., Lepingle, A., Caboche, M. and Vaucheret, H. (1993) Mol. Gen. Genet. 236, 203–208.
[34] Friemann, A., Brinkmann, K. and Hachtel, W. (1992) Mol. Gen. Genet. 231, 411–416.
[35] Luque, I., Flores, E. and Herrero, A. (1993) Plant Mol. Biol. 21, 1201–1205.
[36] Suzuki, I., Sugiyama, T. and Omata, T. (1993) Plant Cell Physiol. 34, 1311–1320.
[37] Peck, H.D. and LeGall, J. (1982) Phil. Trans. R. Soc. London, B298, 443–466.