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REACTIONS OF THE *NEUROSPORA CRASSA* NITRATE REDUCTASE WITH NAD(P) ANALOGS

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Summary

The assimilatory NADPH-nitrate reductase (NADPH:nitrate oxidoreductase, EC 1.6.6.3) from *Neurospora crassa* is competitively inhibited by 3-aminopyridine adenine dinucleotide (AAD) and 3-aminopyridine adenine dinucleotide phosphate (AADP) which are structural analogs of NAD and NADP, respectively. The amino group of the pyridine ring of AAD(P) can react with nitrous acid to yield the diazonium derivative which may covalently bind at the NAD(P) site. As a result of covalent attachment, diazotized AAD(P) causes time-dependent irreversible inactivation of nitrate reductase. However, only the NADPH-dependent activities of the nitrate reductase, i.e. the overall NADPH-nitrate reductase and the NADPH-cytochrome *c* reductase activities, are inactivated. The reduced methyl viologen- and reduced FAD-nitrate reductase activities which do not utilize NADPH are not inhibited. This inactivation by diazotized AADP is prevented by 1 mM NADP. The inclusion of 1 μM FAD can also prevent inactivation, but the FAD effect differs from the NADP protection in that even after removal of the exogenous FAD by extensive dialysis or Sephadex G-25 filtration chromatography, the enzyme is still protected against inactivation. The FAD-generated protected form of nitrate reductase could again be inactivated if the enzyme was treated with NADPH, dialyzed to remove the NADPH, and then exposed to diazotized AADP. When NADP was substituted for NADPH in this experiment, the enzyme remained in the FAD-protected state.

Difference spectra of the inactivated nitrate reductase demonstrated the presence of bound AADP, and titration of the sulfhydryl groups of the inactivated enzyme revealed that a loss of accessible sulfhydryls had occurred.

Abbreviations: $FADH_2$, reduced FAD; AAD, 3-aminopyridine adenine dinucleotide; AADP, 3-aminopyridine adenine dinucleotide phosphate; AAD(P) refers collectively to AAD and/or AADP; Nbs_2, 5,5′-dithiobis(2-nitrobenzoic acid).

The hypothesis generated by these experiments is that diazotized AADP binds at the NADPH site on nitrate reductase and reacts with a functional sulfhydryl at the site. FAD protects the enzyme against inactivation by modifying the sulfhydryl. Since NADPH reverses this protection, it appears the modifications occurring are oxidation-reduction reactions. On the basis of these results, the physiological electron flow in the nitrate reductase is postulated to be from NADPH via sulfhydryls to FAD and then the remainder of the electron carriers as follows:

NADPH → -SH → FAD → cytochrome *b*-557 → Mo → NO_3^-

Introduction

The electron carriers in the electron transport pathway of the *Neurospora crassa* assimilatory nitrate reductase (NADPH:nitrate oxidoreductase, EC 1.6.6.3) have been determined to be:

NADPH → FAD → cytochrome *b*-557 → Mo → NO_3^- (1)

Partial functions of the overall electron transport pathway can be assayed: (A) by using mammalian cytochrome *c* as an alternative electron acceptor in an FAD-dependent reaction in which the molybdenum moiety of the enzyme is not involved (NADPH-cytochrome *c* reductase activity); or (B) by using electron donors such as reduced methyl viologen or reduced FAD ($FADH_2$) to assay the functions of the nitrate-reducing terminus of the enzyme apart from its NADPH-dependent activities (reduced methyl viologen- or $FADH_2$-nitrate reductase activities). However, the initial reaction of NADPH binding and electron transfer to the enzyme has never been closely examined. Inhibition studies with *p*-hydroxymercuribenzoate have implicated one or more essential sulfhydryl groups for the NADPH-dependent activities of nitrate reductase [1], but no indication of the specific localization of function of these sulfhydryl groups on the enzyme has been shown.

A way to study the functional significance of these sulfhydryls may now be available. Anderson and coworkers [2,3] have synthesized 3-aminopyridine adenine dinucleotide (AAD) and 3-aminopyridine adenine dinucleotide phosphate (AADP) analogs of NAD and NADP which differ from NAD and NADP in that the carboxamido group at the 3-position of the pyridine ring is replaced by an amino group (Fig. 1). They have further demonstrated that AAD or AADP are coenzyme-competitive inhibitors of several NAD- or NADP-requiring enzymes [2,3]. The specificity of inhibition by AAD or AADP parallels the preference of the pyridine nucleotide-dependent enzyme for NAD or NADP.

The amino group of the AAD(P) can be reacted with nitrous acid to yield the diazonium derivative [2] (Fig. 1). Diazotized AAD(P) was shown to react with the sulfhydryl groups of compounds such as cysteine, mercaptoethanol, and glutathione [4]. An interesting property of these diazotized AAD(P) derivatives is that they may act as site-labeling reagents by reacting covalently with sulfhydryl groups at the NAD(P) binding sites of enzymes. Diazotized AAD caused pseudo first-order irreversible inactivation of yeast alcohol dehy-

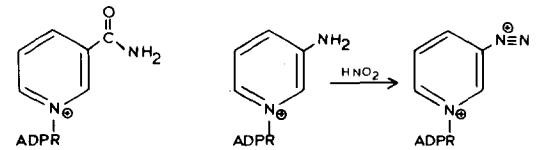

Fig. 1. Comparison of the structures of NAD(P), AAD(P), and the diazotized derivative of AAD(P). ADPR, adenosine diphosphoribose.

drogenase, and the rate of inactivation was decreased by the presence of NAD [2]. Another pyridine derivative, 1-methyl-3-amino-pyridinium chloride, was also diazotized and tested on yeast alcohol dehydrogenase. However, this reagent which carries no ADP-ribose moiety was only one-tenth as inhibitory as diazotized AAD, indicating that the degree of inactivation was directly related to the degree of recognition between the diazotized coenzyme analogs and the NAD(P) site [4]. Nbs_2 titration of the sulfhydryl groups on the yeast alcohol dehydrogenase after inactivation by diazotized AAD showed the loss of one sulfhydryl group per catalytic site. Acid hydrolysis of the inactivated enzyme and subsequent analysis of the hydrolysate identified one cysteine residue per catalytic site which had become covalently linked to the 3-position of a pyridinium ring contributed by the diazotized AAD. Thus, diazotized AAD was shown to be an NAD site-directed agent that specifically interacted with a sulfhydryl side chain of a cysteine residue present at the active site of yeast alcohol dehydrogenase.

The corresponding NADP analog, AADP, was demonstrated to be a competitive inhibitor with respect to NADP in reactions catalyzed by five NADP-requiring enzymes [3]. However, diazotized AADP did not cause a time-dependent irreversible inactivation of these enzymes, possibly because there is no sulfhydryl group present at the coenzyme binding site of these enzymes for covalent attachment of diazotized AADP to occur.

The present study was initiated to investigate the nature of the interaction of the pyridine nucleotide analogs with the NADPH binding site of the *N. crassa* NADPH-nitrate reductase. We asked these questions: Does AAD or AADP compete with NADPH? Does diazotized AADP cause time-dependent inactivation of the nitrate reductase? What information would an NADPH site-specific reagent yield concerning the electron transfer reactions of this enzyme?

A preliminary account of this research has been presented [5].

Methods

N. crassa wild type mycelia (5297a) were grown and the nitrate reductase enzyme preparations were purified essentially as described by Garrett and Nason [1], except that the Sephadex G-200 column chromatography and all subsequent procedures were carried out using phosphate buffers, pH 6.0, from which mercaptoethanol was omitted. The nitrate reductase was further purified by affinity chromatography on Dextran Blue-Sepharose prepared according to

Ryan and Vestling [6]. The enzyme was applied to a column (0.9 × 8 cm) equilibrated with 10 mM phosphate buffer, pH 6, containing 1 mM EDTA. The column was washed with 5—10 volumes of the same buffer. Nitrate reductase was eluted with one column volume of 0.5 mM NADPH in the same buffer. Specific activities attained ranged from $1 \cdot 10^4$ to $4 \cdot 10^4$ nmol of nitrate reduced/min per mg protein. The purest fractions yet reported have specific activities of 10^5 nmol of nitrate reduced/min per mg protein [7].

NADPH-nitrate reductase activity was measured by following the rate of oxidation of NADPH at 340 nm [1]. The assays for NADPH-cytochrome *c* reductase, reduced methyl viologen- , and reduced FAD-nitrate reductase activities were described previously [1]. Activity units reported here are nmol of substrate transformed/min. Kinetic data were obtained on a Gilford 2400 or Cary 14 recording spectrophotometer. The double reciprocal Lineweaver-Burk plots for the determination of K_m and K_i values were drawn according to coordinates obtained from analysis of the data by the least squares method using a Wang Series 370 electronic calculator. Difference spectra were measured at room temperature in cuvettes of 1-cm light path on a Cary Model 14 recording spectrophotometer equipped with 0—0.1 or 0—1 absorbance-sensitive slide-wires.

The 3-aminopyridine adenine dinucleotide (AAD) was prepared from NAD by the Hofmann hypobromite reaction [2]; and 3-aminopyridine adenine dinucleotide phosphate (AADP) was synthesized from NADP and 3-aminopyridine by the pig brain nicotinamide adenine dinucleotide nucleosidase-catalyzed pyridine base exchange reaction [3]. The concentration of AAD or AADP was determined from the absorbance at 331 nm using a molar extinction coefficient of $3.09 \cdot 10^3$ [3].

The diazotization of AAD or AADP was performed according to Fisher et. al. [2]. All reactions were carried out at 0—4°C. In a typical experiment, 0.2 ml of 0—10 mM AAD or AADP was mixed with 0.05 ml of 1 M HCl, followed by 0.1 ml of 0.1 M $NaNO_2$. After incubation for 10 min, 0.1 ml of 0.2 M ammonium sulfamate was added slowly with mixing in order to destroy the excess nitrous acid. After an additional 10 min, the solution was neutralized with 0.05 ml of 1 M NaOH and 0.1 M potassum phosphate buffer, pH 7.0. Such solutions served as the source of diazotized AAD(P) and were used for treatment of the nitrate reductase. Generally, diazotized AAD(P) solution and enzyme were mixed to give a final reaction volume of 1 ml. Aliquots were then removed at various time intervals and enzyme activity was determined by the standard assays.

The number of sulfhydryl groups on the enzyme was determined by titration with 4,4′-dithiodipyridine [8]. The reactions were performed at room temperature using 0.1 M potassium phosphate buffer, pH 7, and the increase in absorbance at 324 nm was measured on a Gilford spectrophotometer. The reactions with the nitrate reductase were complete in 90 min. A standard curve was determined with reduced glutathione. Protein concentration was measured according to the method of Lowry et al. [9] with crystalline bovine serum albumin as the standard.

NAD, NADH, NADP, NADPH, FAD, and cytochrome *c* were obtained from Sigma Chemical Co. The 4,4′-dithiodipyridine (Aldrithiol-4) was purchased from Aldrich Chemical Co. All other chemicals were reagent grade from Fisher or Baker.

Results

Inhibition by AAD or AADP

Although the nitrate reductase from *N. crassa* can use either NADH or NADPH as an electron donor in vitro, NADPH is considered to be the physiological electron donor in vivo because it demonstrates a 3-fold lower K_m and approximately a 5-fold greater V [1]. As demonstrated in the Lineweaver-Burk plot of Fig. 2, both AAD and AADP are competitive inhibitors with respect to NADPH. The K_i for AADP, 0.3 mM, is approximately three times less than the K_i for AAD, 0.85 mM, a situation which parallels the relative K_m values of this enzyme for NADPH and NADH.

Inactivation by diazotized AAD or AADP

When nitrate reductase was incubated with the diazotized derivative of either AAD or AADP, there was a time-dependent inactivation of the enzyme (Fig. 3). Activity is plotted on a log scale and the inactivation process can be fitted to pseudo first-order kinetics when excess diazotized AADP is present. The control solutions lacked only AADP and showed no loss of activity during the same treatment. Comparable levels of inactivation are obtained with much lower concentrations of diazotized AADP than diazotized AAD, reflecting both the increased velocity and increased affinity of the enzyme for NADPH relative to NADH, as cited above.

In order to investigate the nature of the enzyme inactivation caused by the diazotized AADP, its effect on the different portions of the electron transport pathway were determined by assaying the various activities associated with

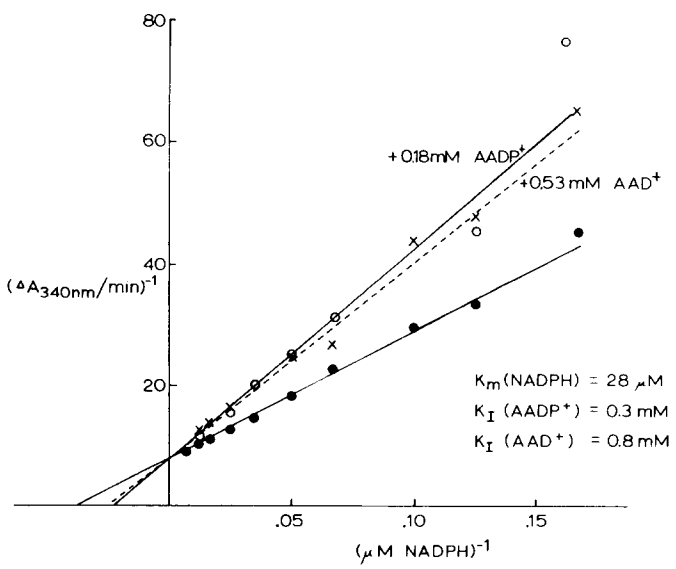

Fig. 2. A typical Lineweaver-Burk plot showing competitive inhibition of nitrate reductase by AAD and AADP with respect to NADPH. Experimental conditions were as in the standard assay for the NADPH-nitrate reductase except that the concentration of NADPH was varied. ●———●, control; X———X, 0.18 mM AADP; ○———○, 0.53 mM AAD.

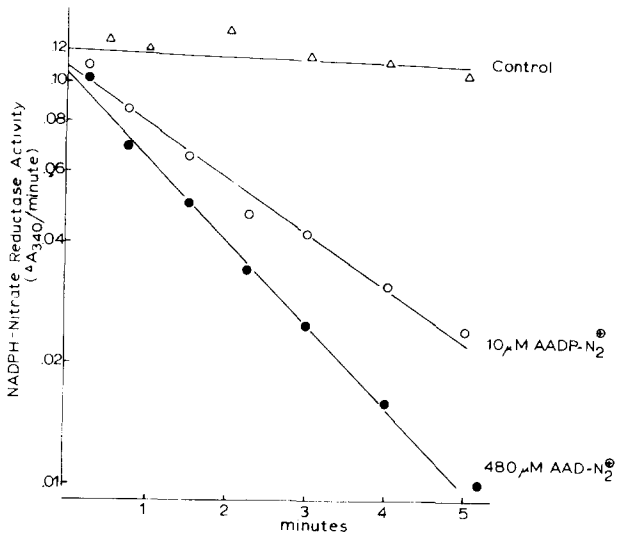

Fig. 3. Time-dependent inactivation of nitrate reductase by diazotized AAD or diazotized AADP displaying pseudo first-order decay. Activity is plotted on a log scale. The diazotized AAD(P) was prepared as described in Materials and Methods and used immediately. △———△, control; ○———○, 10 μM diazotized AADP; ●———●, 480 μM diazotized AAD.

nitrate reductase (Table I). Both NADPH-nitrate reductase and the associated NADPH-cytochrome *c* reductase were inactivated at the same rate (not shown) and to the same extent by diazotized AADP. However, the two other associated

TABLE I

DIFFERENTIAL INACTIVATION OF THE NADPH-NITRATE REDUCTASE AND ASSOCIATED ACTIVITIES

Nitrate reductase was incubated with 72 μM diazotized AADP for 30 min at 0°C, then assayed. The enzyme sample was dialyzed overnight against several changes of 10 mM phosphate buffer, pH 6.0, with 1 mM EDTA, then assayed again. The minus-AADP control enzyme was treated identically except that AADP was omitted from the diazotization reaction

Treatment	Specific activity			
	NADPH-nitrate reductase *	NADPH-cytochrome *c* reductase **	Reduced methyl viologen-nitrate reductase ***	$FADH_2$-nitrate reductase ***
1. Untreated	2058	10 364	2544	603
2. 72 μM diazotized AADP-nitrate reducase	117	1 176	na	na
3. Dialyzed diazotized nitrate reductase	129	758	2595	613
4. Minus-AADP control nitrate reductase	2500	12 044	na	na
5. Dialyzed control nitrate reductase	2250	11 309	2925	583

* nmol NADPH oxidized · min^{-1} · mg^{-1}

** nmol cytochrome *c* reduced · min^{-1} · mg^{-1}.

*** nmol nitrite formed · min^{-1} · mg^{-1}.

na., This enzyme sample could not be assayed because the components of the diazotization mixture interfered with the assay for nitrite.

activities of the nitrate reductase which are not involved with NADPH oxidation, i.e. the reduced methyl viologen-nitrate reductase and $FADH_2$-nitrate reductase, were unaffected. When nitrate reductase was incubated with 57 μM diazotized AADP for 30 min, 90—95% of both NADPH-dependent activities were lost (line 2). The reduced methyl viologen- and $FADH_2$-nitrate reductase activities could not be assayed immediately because the excess free diazotized AADP and sulfamate interfere with the colorimetric assay for nitrite. After dialysis to remove the interfering substances, it can be seen that the reduced methyl viologen- and $FADH_2$-nitrate reductase activities were not inactivated (line 3). It is significant to note that the NADPH-dependent activities remain inactivated after dialysis. When nitrate reductase was incubated with a complete diazotization mixture lacking only AADP (line 4), none of the activities were affected and the enzyme was stable to dialysis (line 5).

NADP(H) protection against inactivation by diazotized AADP

Further studies were then performed to support the notion that the diazotized AADP was acting at the NADPH-binding site of nitrate reductase rather than exerting a non-specific inhibition. When 1 mM NADP or 1 mM NADPH was added to the enzyme at 0°C along with the diazotized AADP, NADPH-nitrate reductase activity was protected against inactivation (Fig. 4). The NADPH-cytochrome *c* reductase activity was also protected (not shown). Smaller amounts of NADP protected to a lesser extent. When the enzyme was treated with NADP or NADPH and then dialyzed, the enzyme was again susceptible to inactivation. The addition of 1—10 mM $NaNO_3$ during treatment of the nitrate reductase with the diazotization mixture did not have any effect.

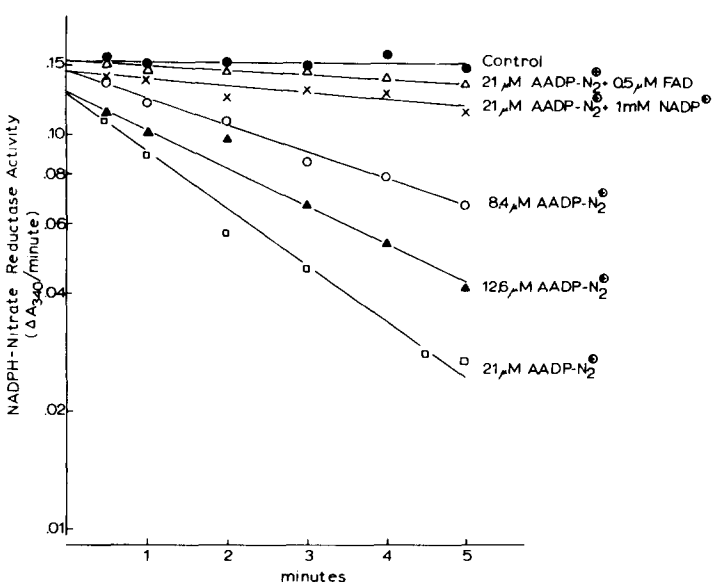

Fig. 4. Time-dependent inactivation of nitrate reductase by diazotized AADP: Protection by NADP or FAD. Activity is plotted on a log scale. The 0.5 μM FAD or 1 mM NADP was preincubated with the enzyme for 10 min before addition of the 21 μM diazotized AADP.

FAD protection against inactivation by diazotized AADP

FAD is an essential cofactor of the nitrate reductase but is readily lost from the enzyme during purification and hence must be added to assay mixtures in order to obtain enzymatic activity [1,10]. Interestingly, when FAD was added to the inactivation mixture, the enzyme was protected effectively against inactivation by diazotized AADP. Maximal protection with minimal flavin concentrations was attained by briefly preincubating the enzyme with the FAD before treatment with the diazotized AADP. For example, when nitrate reductase was preincubated with 0.5 μM FAD for 10 min at 0°C before addition of 21 μM diazotized AADP, no inactivation occurred. As seen in the Lineweaver Burk plot (Fig. 5), inhibition by AADP was non-competitive with respect to FAD. The K_i for AADP is 0.6 mM. The K_m of the enzyme for FAD is 90 nM.

As shown in Table II, when nitrate reductase was preincubated with 5 μM FAD, then either extensively dialyzed (line 3) or passed through a Sephadex G-25 column (line 4) to remove the exogenous FAD, the enzyme was still protected against inactivation by diazotized AADP. Thus, the FAD-induced protected state of the enzyme persisted even after the exogenous FAD was removed. When nitrate reductase was treated with 5 μM FAD, then dialyzed, treated with 1 mM NADPH, then chromatographed through Sephadex G-25 to remove the NADPH (line 5), the enzyme was returned to a state susceptible to inactivation by diazotized AADP. On the other hand, when 1 mM NADP was substituted for NADPH in the treatment (line 6), the protected form generated by FAD treatment persisted and the enzyme was not inactivated by diazotized AADP. Dithionite, used instead of NADPH in a similar sequence of addition and removal, could partially overcome the protective state caused by FAD, but the sulfhydryl reagents mercaptoethanol or dithiothreitol (not shown) did not.

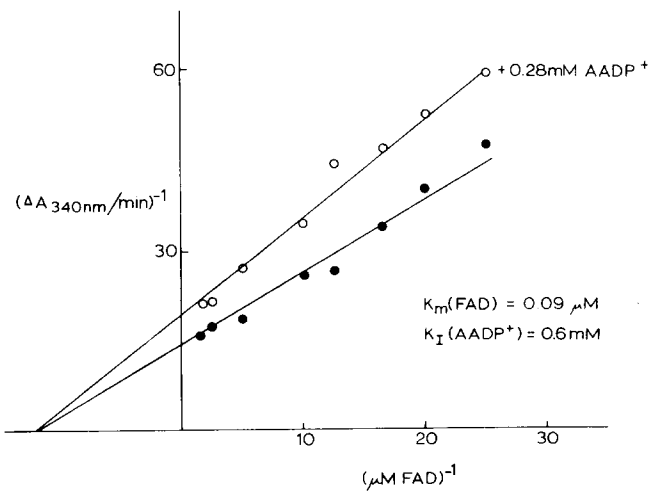

Fig. 5. A Lineweaver-Burk plot demonstrating non-competitive inhibiton of nitrate reductase by AADP with respect to FAD. Nitrate reductase enzyme aliquots were preincubated with the indicated amount of FAD for 5 min at 0°C before addition of the remaining mixture and the 0.8 mM AADP. The reaction was initiated by the addition of NADPH. o———o, 0.28 mM AADP; •———•, control.

TABLE II

FAD-MEDIATED PROTECTION OF NITRATE REDUCTASE FROM INACTIVATION BY DIAZOTIZED AADP

Nitrate reductase was treated with 5 μM FAD for 30 min at 0°C. The enzyme was then treated with 36 μM diazotized AADP either immediately (line 2) or after removal of the FAD by dialysis against 10 mM phosphate buffer, pH 6.0, with 1 mM EDTA (line 3) or by Sephadex G-25 column chromatography (line 4) in the same buffer. Aliquots of FAD-treated, dialyzed enzyme were incubated with either 1 mM NADPH, 1 mM NADP or 1 mM dithionite for 30 min at 0°C. These reagents were removed from the sample by Sephadex G-25 chromatography and then the enzyme was treated with diazotized AADP. Control enzyme preparations were treated identically except that the AADP was missing from the inactivation mixture.

Treatment before incubation with 36 μM diazotized AADP	Percent initial NADPH-nitrate reductase remaining after 5 min incubation with diazotized AADP
No treatment	25
5 μM FAD	100
5 μM FAD → dialysis	100
5 μM FAD *	95
5 μM FAD → dialysis → 1 mM NADPH *	25
5 μM FAD → dialysis → 1 mM NADP *	82
5 μM FAD → dialysis → 1 mM dithionite *	35
5 μM FAD → dialysis → 1 mM mercaptoethanol *	90

* Enzyme subjected to Sephadex G-25 chromatography after the indicated treatment.

Further experiments to establish the mechanism and assumed involvement of sulfhydryls were carried out. The ultraviolet absorption spectra of native and diazotized AADP-treated nitrate reductase are shown in Fig. 6. The difference spectra is also shown and it compares favorably with the known absorption spectrum of AAD(P) [2]. By assuming the molar extinction coefficient of diazotized AADP at 262 nm is the same for the enzyme-bound form as for the free form [2], it was possible to calculate the amount of AADP bound to the enzyme. Approximately three diazotized AADP residues were bound per heme. The amount of heme in the nitrate reductase was determined by the absorbance of the oxidized enzyme at 412 nm, using the molar extinction coefficient of $137 \cdot 10^3$. The amount of diazotized AADP bound is expressed per heme

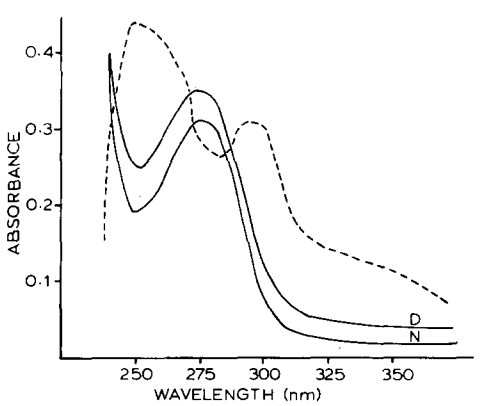

Fig. 6. Absorption spectra of native (N) and diazotized AADP-modified (D) nitrate reductase in 0.1 M phosphate buffer, pH 7.0. The difference spectra (- - - - -) is plotted magnified X 10.

because of the varying amounts of contaminating protein even in the most purified preparations.

A comparison of the number of titratable sulfhydryl groups in the native and diazotized enzyme was undertaken in order to determine whether the binding of the diazotized AADP caused the loss of sulfhydryl as occurred in the yeast alcohol dehydrogenase. By titration with 4,4′-dithiodipyridine, 46 sulfhydryls per heme were determined for the control nitrate reductase preparation, and 42 sulfhydryls per heme for the nitrate reductase inactivated by diazotized AADP. The values for the total sulfhydryls may not accurately represent the sulfhydryl content of the nitrate reductase because of contaminating protein in the preparations. However, this sulfhydryl determination does show unmistakably that there is a loss of accessible sulfhydryl groups in the nitrate reductase after the binding of diazotized AADP and that the number of sulfhydryls lost per heme correlates well with the number of AADP molecules per heme as determined by the difference spectra.

We attempted to isolate the pyridyl-cysteine derivative of diazotized AADP-treated nitrate reductase after acid hydrolysis of the inactivated enzyme in the manner described by Chan and Anderson [4]. However, the large phenylalanine peak obtained in the amino acid analysis so obscured the region in which the *S*-3-pyridylcysteine was also expected to elute that no definitive statement could be made concerning the presence of AADP bound to a cysteine residue.

Discussion

AAD or AADP competitively inhibits the nitrate reductase from *N. crassa*. Inhibition of NAD(P) dehydrogenases by these pyridine nucleotide analogs appears to reflect the relative specificity of such enzymes for the two naturally occurring coenzymes, NAD or NADP. For example, the NAD-dependent yeast alcohol dehydrogenase is inhibited by AAD (the NAD analog) whereas the NADP-specific yeast glucose-6-phosphate dehydrogenase is sensitive only to the NADP analog, AADP [2,3]. The nitrate reductase which can use either NADPH or NADH is inhibited by either analog. Nitrate reductase from *N. crassa* does bind NADPH with a greater affinity and with it attains a higher maximal reaction velocity than with NADH. This situation is reflected by the greater inhibition by AADP relative to AAD.

The diazotized derivatives of both AAD and AADP inactivate nitrate reductase. This inactivation appears to be irreversible since removal of the excess diazotized coenzyme analog by either dialysis or Sephadex G-25 gel filtration chromatography does not lead to any recovery of enzyme activity. Again, on a concentration basis, diazotized AADP exerts much greater inactivation. Not every enzyme that is competitively inhibited by AAD(P) can be irreversibly inactivated by the diazotized derivative [3]. Since the diazotized derivative appears to react specifically with sulfhydryl groups at neutral pH [4], perhaps the enzyme must have accessible sulfhydryl groups at the coenzyme binding site which are close enough to the diazo function of the pyridine moiety of AAD(P) to react in order for inactivation to occur.

Only the two NADPH-dependent activities of the nitrate reductase, namely

the NADPH-nitrate reductase and the NADPH-cytochrome *c* reductase activities, were inhibited by the diazotized AADP. Further, only these two activities of the nitrate reductase are inhibited by low concentrations (5 μM) of the sulfhydryl binding agent, *p*-hydroxymercuribenzoate [1]. Evidence from the difference spectra revealed the presence of diazotized AADP bound to the enzyme, and subsequent titration of accessible sulfhydyls revealed a concomitant loss of sulfhydryls in the diazotized AADP-treated nitrate reductase compared to the control enzyme preparation. The value of three AADP bound per nitrate reductase heme may be high. One explanation for this apparent discrepancy is the presence of unknown NAD(P)H dehydrogenases in the final nitrate reductase preparations which might also bind diazotized AAD(P). Indeed, the last purification step used, elution of the nitrate reductase from the Dextran Blue-Sepharose affinity column by NADPH, would select for such contaminations.

Micromolar amounts of FAD protect nitrate reductase against inactivation by diazotized AADP. After FAD treatment, even when all the exogenous FAD was removed from the enzyme preparation by extensive dialysis, the nitrate reductase was still protected against inactivation. This suggests that the FAD might be modifying the enzyme and thereby altering its susceptibility to inhibition by diazotized AADP rather than interfering directly with the action of the inhibitor. FAD shows non-competitive interaction with respect to the inhibition of the nitrate reductase by AADP. The simplest explanation is that FAD binds to the enzyme at a site distinct from the NADPH binding site. Yet, the FAD protects against inactivation by modifying the enzyme so that the postulated sulfhydryl group at the NADPH binding site is no longer accessible or reactive towards the covalent attachment of the diazotized AADP.

Despite the apparent linearity of Figs. 3 and 4, the nitrate reductase, as prepared in the usual purification steps, displays a rate of inactivation by diazotized AADP that decreases progressively and usually only about 80—90% of the enzyme can be inactivated even after prolonged incubation with diazotized AAD(P). However, when the enzyme is treated with NADPH, then dialyzed, the inactivation by diazotized AADP proceeds linearly throughout, until all the enzyme is inactivated. Thus, the normal enzyme preparation which was obtained through several ammonium sulfate precipitations, ion-exchange columns, and Sephadex G-200 gel chromatography probably contained from 10 to 20% of the nitrate reductase in the form that was resistant to inactivation, i.e. like the form resulting from FAD treatment. Further, the protective effect generated by FAD can be partially overcome by treatment with dithionite. Other oxidizing or reducing or sulfhydryl agents did not overcome the FAD protection.

At least two explanations for the persistent FAD modification of the nitrate reductase can be considered. One is that the FAD may bind tightly to the enzyme and physically shield the postulated sulfhydryl group to make it inaccessible to the diazonium. If so, this tightly bound FAD is resistant to removal from the enzyme by prolonged dialysis, because the FAD-treated and dialyzed enzyme is not readily inactivated by the diazotized AADP. For this alternative to be correct, NADPH but not $NADP^+$ must trigger a release of the FAD from the nitrate reductase since NADPH treatment of the enzyme subsequent to

FAD addition and removal by dialysis will render the nitrate reductase susceptible to inactivation again. Thus, either NADPH specifically, or the reduction reaction initiated by it, would lead to FAD release and a consequent exposure of functional groups, presumably cysteine sulfhydryls, which diazotized AADP could attack once the NADPH was removed.

A second and perhaps more satisfactory explanation is that FAD may exert a redox change in the enzyme to cause a sulfhydryl-disulfide transition. There is precedent for such a reaction mechanism with a number of enzymes, such as glutathione reductase and lipoyl dehydrogenase [11,12]. In this explanation, it is assumed that in the nitrate reductase as isolated routinely, the sulfhydryls would be predominately (80—90%) in the reduced state. This reduced state might be expected since most of the nitrate reductase purification procedure is carried out with the enzyme in the presence of 1 mM mercaptoethanol. Our hypothesis is that the reduced sulfhydryls of the nitrate reductase (form I in Fig. 7) are susceptible to covalent attack by diazotized AADP whereas the suggested disulfide state (form II) is unreactive to the diazonium. FAD could cause the formation of the disulfide from the sulfhydryls (i.e. a transition from form I to form II via reactions 1 and 2, Fig. 7). As suggested, this disulfide would be insensitive to reaction with diazotized AADP and the enzyme would be protected. NADPH would reverse the FAD effect by reducing once more the disulfide to sulfhydryls so that these groups would then be available to react with diazotized AADP. In this hypothesis, a disulfide is postulated only for the sake of simplicity; any oxidized form of a sulfhydryl that would be non-reactive with diazotized AAD(P) would be consistent with this interpretation.

If the hypothesis we favor is correct, then the disulfide-sulfhydryl change may represent the initial redox reaction that occurs between the the electron donor NADPH and the nitrate reductase perhaps as represented by reactions 3 and 4. FAD would function to transfer the reducing equivalents introduced by NADPH further along the electron transport chain to cytochrome *b*-557 and, ultimately, nitrate is reduced. Thus, these experiments suggest the sequence of

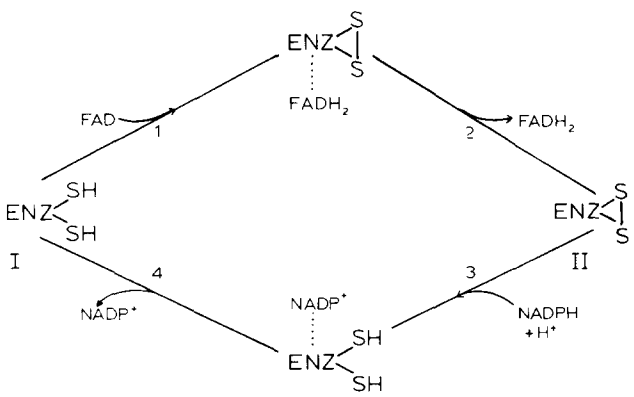

Fig. 7. Proposed mechanism of nitrate reductase for the initial electron transfer reactions from NADPH. Form I is postulated to be susceptible to inhibition by diazotized AADP, whereas form II would be insensitive to inhibition.

electron transfer mediated by the nitrate reductase might be best described as:

NADPH → -SH → FAD → cytochrome *b*-557 → Mo → NO_3^-.

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