Journal of Bioenergetics and Biomembranes, Vol. 17, No. 1, 1985

Studies of the Ferricyanide Reductase Activities of the Mitochondrial Reduced Nicotinamide Adenine Dinucleotide-Ubiquinone Reductase (Complex I) Utilizing Arylazido-β-alanyl NAD^+ and Arylazido-β-alanyl $NADP^+$

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Received January 30, 1984; revised June 11, 1984

Abstract

The NADH and NADPH ferricyanide reductase activities present in mitochondrial NADH-CoQ reductase preparations have been studied utilizing two photoaffinity pyridine nucleotide analogues: arylazido-β-alanyl NAD^+ (A3′-O-{3-[*N*-(4-azido-2-nitrophenyl)amino]propionyl}NAD^+) and arylazido-β-alanyl $NADP^+$ (N3′-O-{3-[*N*-(4-azido-3-nitrophenyl)amino]-propionyl}$NADP^+$). For the NADH-$K_3Fe(CN)_6$ reductase activity, arylazido-β-alanyl NAD^+ was found to be, in the dark, a competitive inhibitor with respect to both NADH and $K_3Fe(CN)_6$ with $K_{i,app}$ values of 9.7 and 15.5 μM, respectively. In comparison the $NADP^+$ analogue exhibited weak noncompetitive inhibitor activity for this reaction against both substrates. Upon photoirradiation arylazido-β-alanyl NAD^+ inhibited NADH-$K_3Fe(CN)_6$ reductase up to 70% in the presence of a 25-fold molar excess of analogue over the enzyme concentration. This photodependent inhibition could be prevented by the presence, during irradiation, of the natural substrate NADH. In contrast complex kinetic results were obtained with studies of the effects of the pyridine nucleotide analogues of NADPH-$K_3Fe(CN)_6$ reductase activity in the dark. Photoirradiation of either analogue in the presence of the enzyme complex resulted in an activation of NADPH-dependent activity. The possibility that the NADPH-$K_3Fe(CN)_6$ reductase activity of complex I represents a summation of the combined ferricyanide reductase activity of the NADPH-NAD^+ transhydrogenase and NADH oxidoreductase is suggested.

Key Words: NADH ferricyanide reductase; NADPH ferricyande reductase; photoaffinity; pyridine nucleotide analogues; arylazido-β-alanyl NAD^+; arylazido-β-alanyl $NADP^+$; reduced nicotinamide adenine dinucleotide-ubiquinone reductase; complex I; NADPH-NAD^+ transhydrogenation; NADH oxidoreductase.

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0145-479X/85/0200-0033$04.50/0

Introduction

The photoaffinity analogues of the pyridine nucleotides, arylazido-β-alanyl NAD^+ and arylazido-β-alanyl $NADP^+$,[2] have been utilized in studies of the relationship between the various pyridine nucelotide-dependent activities present in the mitochondrial complex I, NADH-CoQ reductase (Chen and Guillory, 1979, 1980). This enzyme complex can utilize both NADH and NADPH as electron donors for CoQ reduction (Hatefi and Hanstein, 1973). In addition, the preparation catalyzes both forward and reverse pyridine nucleotide transhydrogenase activities (Hatefi and Hanstein, 1973). Utilizing the site-specific photodependent labeling properties of the two pyridine nucleotide analogues, our previous studies revealed that both NADH and NADPH are oxidized at an identical site during oxidoreduction reactions (Chen and Guillory, 1979, 1980). The latter studies also indicated that the two pyridine nucleotide transhydrogenase activities (NADH-AcPyAD$^+$ and NADPH-AcPyAD$^+$ transhydrogenations) associated with complex I were catalyzed at two separate sites, both of which are distinct from the reactive site for NAD(P)H-CoQ reductase activity. These analogues have now been tested on the artificial dehydrogenase activities, NADH-$K_3Fe(CN)_6$ and NADPH-$K_3Fe(CN)_6$ reductase. Our analysis indicates that the NADPH-$K_3Fe(CN)_6$ reductase activity is a consequence of the ferricyanide reductase activity of the oxidoreductase and the NADPH-NAD^+ transhydrogenase enzymes. This unexpected result is the subject of this communication.

Materials and Methods

NAD^+, NADH, $NADP^+$, and palmitoyl CoA were obtained from Sigma Chemical Company, NADPH from Calbiochem, NaN_3 from K and K Laboratories, and $K_3Fe(CN)_6$ from Mallinckrodt Chemical Company. Arylazido-β-alanyl NAD^+ and arylazido-β-alanyl $NADP^+$ were prepared as previously described (Chen and Guillory, 1977, 1980).

The NADH-CoQ reductase (complex I) (EC 1.6.5.3) was isolated from ox heart mitochondria according to the procedure described by Hatefi *et al.*, (1962). NADH-$K_3Fe(CN)_6$ reductase activity was measured by the decrease in the absorbance at 420 nm of a solution containing 50 mM phosphate buffer, pH 7.0, 2 mM NaN_3, 80 μM NADH, 480 μM $F_3Fe(CN)_6$, and enzyme complex at 37°C in a final volume of 1 ml. $K_3Fe(CN)_6$ reduction was

[2] Abbreviations used. arylazido-β-alanyl NAD^+, A3′-O-{3-[*N*-(4-azido-2-nitrophenyl)amino]-propionyl}NAD^+; arylazido-β-alanyl $NADP^+$, N3′-O-{3-[*N*-(4-azido-2-nitrophenyl)amino]-propionyl}$NADP^+$; CoQ, coenzyme Q with one isoprenoid unit in the side change; arylazido-β-alanine, 3-[*N*-(4-azido-2-nitrophenyl)-amino]propionic acid; $K_{i,app}$, apparent K_i; $K_{m,app}$, apparent K_m.

quantified using a molar extinction coefficient of 1000 (Minakami *et al.*, 1962). The NADPH-$K_3Fe(CN)_6$ reductase activity was assayed in the same manner except that 200 μM NADPH replaced the NADH. For kinetic studies the substrate or cofactor concentrations were varied as indicated. Photoirradiation was carried out as previously described by Chen and Guillory (1977).

Results

The Effect of Arylazido-β-alanyl NAD$^+$ and Arylazido-β-alanyl NADP$^+$ on NADH-$K_3Fe(CN)_6$ Activity of Complex I

For NADH-$K_3Fe(CN)_6$ reductase, arylazido-β-alanyl NAD$^+$ has been shown to be a competitive inhibitor for both NADH and $K_3Fe(CN)_6$ (Fig. 1A, B). Minakami *et al.* (1962) and Dookjewaard and Slater (1976) have reported that NADH and ferricyanide compete for an identical reactive site

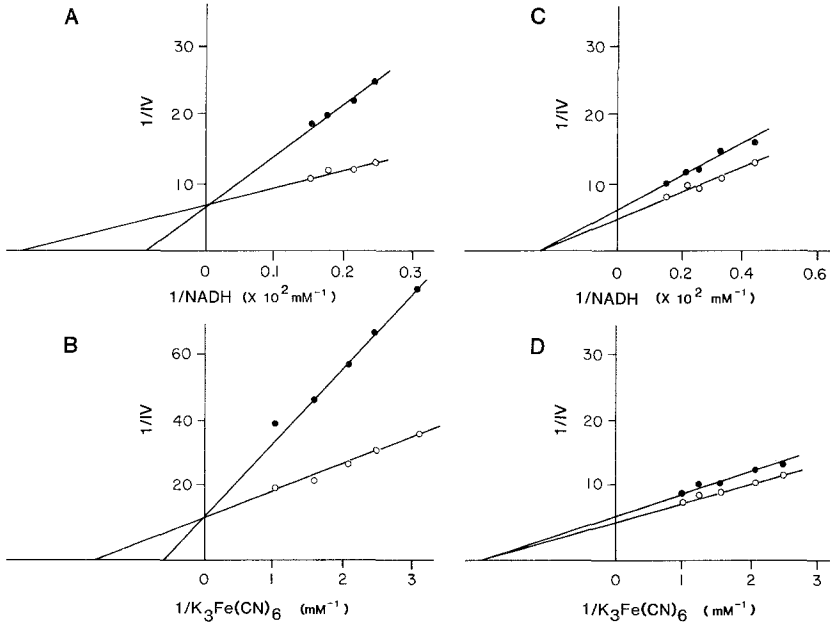

Fig. 1. Kinetic analysis of the arylazido-β-alanyl NAD$^+$ and arylazido-β-alanyl NADP$^+$ inhibition of the NADH-$K_3Fe(CN)_6$ reductase activity of complex I. Enzymatic activity was assayed as described in Materials and Methods. The control activity was assayed at 44.6 μmol $K_3Fe(CN)_6$ reduced/min/mg protein. (A) $K_3Fe(CN)_6$ was 960 μM; 19 μM arylazido-β-alanyl NAD$^+$ (○); control activity (●). (B) NADH was 80 μM; 27 μM arylazido-β-alanyl NAD$^+$ (○); control activity (●). (C) $K_3Fe(CN)_6$ was 480 μM; 193 μM arylazido-β-alanyl NADP$^+$ (○); control activity (●). (D) NADH was 80 μM arylazido-β-alanyl NADP$^+$ (○); control activity (●).

during catalysis of this reaction. Kinetic analysis further indicates that the reaction mechanism for this activity is of a ping pong bi bi order (Dookjewaard and Slater, 1976), the oxidized enzyme being first reduced by NADH. Since ferricyanide is a one-electron acceptor the reduced enzyme must be reoxidized in two one-electron transfer processes. The fact that arylazido-β-alanyl NAD^+ is an effective competitive inhibitor for both substrates is strong support for a mechanism in which NADH and ferricyanide react at the same site.

Arylazido-β-alanyl $NADP^+$ has only a very weak inhibitory effect on the NADH-$K_3Fe(CN)_6$ reductase activity of complex I. Within the limits of its minor inhibitory action it appears from the kinetic data that the analogue acts as a noncompetitive inhibitor with respect to both NADH and $K_3Fe(CN)_6$ (Fig. 1C, D). Maximal inhibition is only of the order of 23 to 24% at 193 μM analogue concentration, suggesting that this inhibition is not directly related to active site interaction.

The Effect of Arylazido-β-alanyl NAD^+ and Arylazido-β-alanyl $NADP^+$ on the NADPH-$K_3Fe(CN)_6$ Reductase Activity of Complex I

The inhibitory influence of the arylazido pyridine nucleotide analogues on the NADPH-$K_3Fe(CN)_6$ reductase activity is more complex than that observed for the NADH-$K_3Fe(CN)_6$ reductase activity. A complicating factor in the analysis of this activity is that NADPH-$K_3Fe(CN)_6$ reductase activity is relatively low, being only one-hundredth of the NADH-dependent activity. It is difficult to assay spectrophotometrically this activity at the low substrate concentration normally used in the NADH-$K_3Fe(CN)_6$ reductase assay. The kinetic results become nonreproducible when the NADPH concentration present during the assay is reduced below 80 μM. A second complication is that while addition of either analogue results in inhibition of NADPH-$K_3Fe(CN)_6$ reductase activity, reciprocal plots of activity against analogue concentration are nonlinear, making it difficult to decide upon the exact kinetic nature of the inhibition (Fig. 2). It is obvious that the pyridine nucleotide analogues inhibit this activity in a more complex fashion than that observed for the NADH-$K_3Fe(CN)_6$ reductase.

Tables I and II summarize the kinetic results obtained on the influence of arylazido-β-alanyl NAD^+ and arylazido-β-alanyl $NADP^+$ on the ferricyanide reductase activities present in complex I.

The Photodependent Effects of the Pyridine Nucleotides and Arylazido-β-alanyl Derivatives on the Ferricyanide Reductase Activities Assayed in Complex I

As shown in Table III, of the two reductase activities assayed, the NADH-$K_3Fe(CN)_6$ reductase is the only activity significantly inhibited by

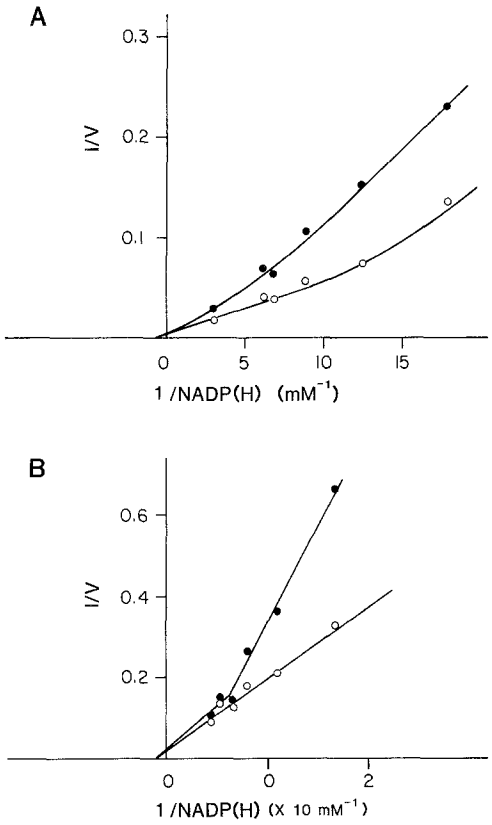

Fig. 2. Kinetic analysis of the arylazido-β-alanyl NAD^+ and arylazido-β-alanyl $NADP^+$ inhibition of the NADPH-$K_3Fe(CN)_6$ reductase activity of complex I. (A) Activity was evaluated at 1.6 μmol $K_3Fe(CN)_6$ reduced/min/mg protein with a $K_3Fe(CN)_6$ concentration of 480 μM (○). With the addition of 26 μM arylazido-β-alanyl NAD^+ (●). (B) As with A, except that the control activity was assayed at 0.89 μmol $K_3Fe(CN)_6$ reduced/min/mg protein (○). With the addition of 48 μM arylazido-β-alanyl $NADP^+$ (●).

arylazido-β-alanyl NAD^+ following photoirradiation. This activity is considered to be representative of the NADH dehydrogenase enzyme. Since we have previously shown that the $NADP^+$ analogue has no effect on the NADH-CoQ reductase activity (Chen and Guillory, 1980), the ineffectiveness of arylazido-β-alanyl $NADP^+$ on the NADH-$K_3Fe(CN)_6$ reductase was

Table I. Effect of Arylazido-β-alanyl NAD^+ on the Ferricyanide Reductase Activities Present in Complex I

Rection	Concentration of fixed substrate (μM)	Concentration of arylazido-β-alanyl NAD^+ (μM)	Inhibitory kinetic effect	$K_{m,app}$ (μM)	$K_{i,app}$ (μM)
NADH-$K_3Fe(CN)_6$	960 ($K_3Fe(CN)_6$)	19	Competitive	35.7 (NADH)	9.7
	80 (NADH)	27	Competitive	606 ($K_3Fe(CN)_6$)	15.5
NADPH-$K_3Fe(CN)_6$	480 ($K_3Fe(CN)_6$)	26	Mixed	1250 (NADPH)	—

Table II. Effect of Arylazido-β-alanyl $NADP^+$ on the Ferricyanide Reductase Activities Present in Complex I

Rection	Concentration of fixed substrate (μM)	Concentration of arylazido-β-alanyl $NADP^+$ (μM)	Inhibitory kinetic effect	$K_{m,app}$ (μM)	$K_{i,app}$ (μM)
NADH-$K_3Fe(CN)_6$	480 ($K_3Fe(CN)_6$)	193	Noncompetitive	42.6 (NADH)	—
	80 (NADH)	193	Noncompetitive	541 ($K_3Fe(CN)_6$)	—
NADPH-$K_3Fe(CN)_6$	480 ($K_3Fe(CN)_6$)	48	Mixed	1180 (NADPH)	—

Table III. Photodependent Effects of the Pyridine Nucleotides and Their Arylazido-β-alanyl Derivatives on the Ferricyanide Reductase Activities Assayed in Complex I

Additions	Percent activity[a]	
	NADH-$K_3Fe(CN)_6$ reductase	NADPH-$K_3Fe(CN)_6$ reductase
1. None	89	94
2. NAD^+	100	110
3. $NADP^+$	93	89
4. Arylazido-β-alanine	87	107
5. NAD^+ + arylazido-β-alanine	94	86
6. $NADP^+$ + arylazido-β-alanine	86	95
7. Arylazido-β-alanyl NAD^+	48	86
8. Arylazido-β-alanyl $NADP^+$	89	140

[a]As compared to the nonphotoirradiated control. Additions were made as indicated at the following concentrations: NAD^+ and $NADP^+$ (4×10^{-8} mol); arylazido-**b**-alanine (4.5×10^{-8} mol); arylazido-β-alanyl NAD^+ (2.6×10^{-8} mol); arylazido-β-alanyl $NADP^+$ (4.4×10^{-8} mol). Photoirradiation was carried out as previously described (Chen and Guillory, 1977) with 0.45 mg of complex I in 0.17 ml. NADH-$K_3Fe(CN)_6$ and NADPH-$K_3Fe(CN)_6$ reductase activities were assayed as described in Materials and Methods and were assayed at 278 μmol $K_3Fe(CN)_6$ and 1.6 μmol $K_3Fe(CN)_6$ reduced min^{-1} mg $protein^{-1}$, respectively. The dark controls containing arylazido-β-alanyl NAD^+ maintained 92 and 93% of the control activity for NADH-$K_3Fe(CN)_6$ and NADPH-$K_3Fe(CN)_6$ reductase activities while those containing arylazido-β-alanyl $NADP^+$ maintained 100% of both activities.

to be expected. The photodependence of the inhibition by arylazido-β-alanyl NAD^+ is evidenced by the negligible inhibition of enzymatic activity for the light controls (i.e., the enzyme photoirradiated in the absence of the analogue) and dark controls (i.e., the enzyme analogue mixture assayed prior to photoirradiation). The specificity of the inhibition due to photoirradiation of complex I in the presence of arylazido-β-alanyl NAD^+ is indicated by the lack of an inhibitory action for NAD^+ and arylazido-β-alanine, alone or when both were present at equimolar quantities. It is obvious that these two components cannot act independently but must be coupled together chemically in order to provide significant inhibition.

NADPH-$K_3Fe(CN)_6$ reductase activity was not inhibited by either pyridine nucleotide analogue. There was in fact a substantial activation following photoirradiation in the presence of arylazido-β-alanyl $NADP^+$. This photodependent activation effect is analyzed below.

The Concentration Dependence for the Photodependent Effects of Arylazido-β-alanyl-NAD^+ and Arylazido-β-alanyl-$NADP^+$ on the Ferricyanide Reductase Activity of Complex I

The NADH-$K_3Fe(CN)_6$ reductase activity was inhibited to a maximum of 70% when complex I was photoirradiated together with arylazido-β-alanyl NAD^+ at a 25-fold molar excess (Fig. 3A). A molecular weight of 570,000 was utilized for the calculation of the complex I concentration (Huennekens and Mackler, 1971). At the same molar ratio arylazido-β-alanyl $NADP^+$ inhibited the NADH-$K_3Fe(CN)_6$ reductase activity by 10%. Fifty-four percent inhibition with the $NADP^+$ analogue could be obtained at concentrations in excess of 400-fold over that of the enzyme complex (Fig. 3B). On the other hand, the NADPH-$K_3Fe(CN)_6$ reductase activity rather than being inhibited was activated by photoirradiation in the presence of either arylazido-β-alanyl NAD^+ or arylazido-β-alanyl $NADP^+$. At a 25-fold molar excess of either reagent over that of the enzyme complex, a twofold activation was observed (Fig. 3C, D).

In Fig. 4A, B, it can be observed that the photodependent inhibition of the NADH-$K_3Fe(CN)_6$ reductase by the pyridine nucleotide analogues can be effectively prevented by the presence of NADH during photoirradiation. The fact that NADPH is not as effective as NADH in protecting against photodependent analogue inhibition is consistent with the relative ability of NADH and NADPH to act as protecting agents against arylazido-β-alanyl NAD^+ photodependent inhibition of NADH-CoQ reductase (Chen and Guillory, 1979). This result supports the suggestion that the NADH-$K_3Fe(CN)_6$ reductase activity is representative of the enzyme catalyzing the NADH-CoQ reductase activity of complex I.

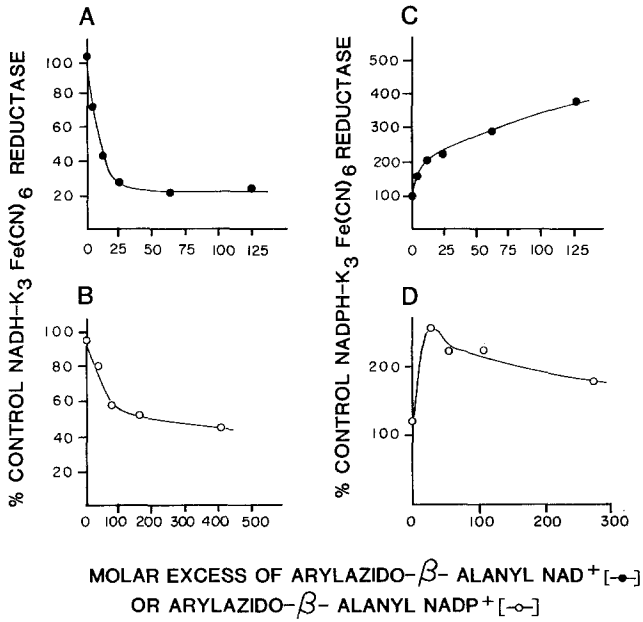

Fig. 3. The photodependent inhibition by arylazido-β-alanyl NAD^+ and arylazido-β-alanyl $NADP^+$ of the ferricyanide reductase activities of complex I. Complex I was assayed prior to and following a 1-min period of light irradiation. That activity prior to photoirradiation was taken as representing 100% activity. Assays were performed as described in Materials and Methods. For NADH-$K_3Fe(CN)_6$ reductase the irradiation mixture contained 1.47×10^{-10} mol of complex I and arylazido-β-alanyl NAD^+ (A) or arylazido-β-alanyl $NADP^+$ (B) at the molar excess over complex I indicated in the respective figures. The final volume was 0.24 mol. For NADPH-$K_3Fe(CN)_6$ reductase, the irradiation mixture contained 4.41×10^{-10} mol of complex I and a concentration of arylazido-β-alanyl NAD^+ (C) or arylazido-β-alanyl $NADP^+$ (D) at a molar excess over complex I as indicated in the legend to the figure. The final volume was 0.18 mol.

In the case of NADPH-$K_3Fe(CN)_6$ reductase activity the interesting photodependent activation in the presence of arylazido-β-alanyl NAD^+ was almost completely prevented when the photoactive analogue was irradiated with the protein in the presence of NADH or NADPH (Fig. 4C). NADH was more effective in preventing the analogue photodependent activation at low concentrations while the effectiveness of both NADH and NADPH at equmolar concentrations with the arylazido derivative were quite similar.

As shown in Fig. 4D, NADPH reverses that photoactivation brought on by arylazido-β-alanyl $NADP^+$, while NADH further potentiates the

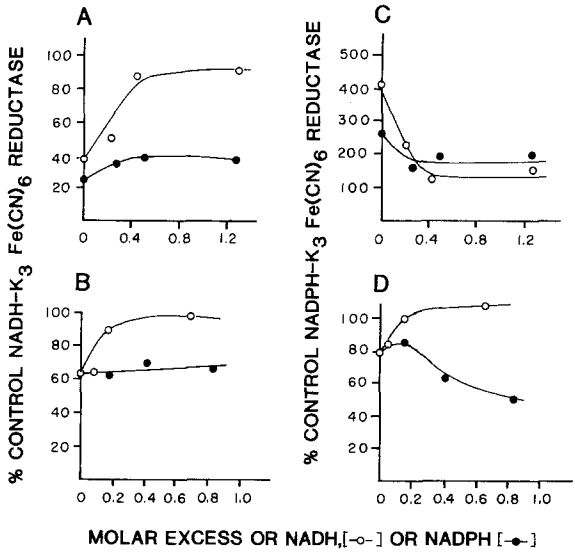

Fig. 4. Pyridine nucleotide protection of the photodependent pyridine nucleotide analogue inhibition of the ferricyanide reductase activities of complex I. All conditions are as described in the legend for Fig. 3 except that: (A) for the NADH-$K_3Fe(CN)_6$ reductase activity, the irradiated mixture contained 1.47×10^{-10} mol of complex I, 3.47×10^{-9} mol of arylazido-β-alanyl NAD^+ and NADH (○) [or NADPH (●)] at the indicated molar excess over that of arylazido-β-alanyl NAD^+. The final volume was 0.24 ml. (B) As with (A) except that the irradiated mixture contained 24.2×10^{-9} mol of arylazido-β-alanyl $NADP^+$ in place of arylazido-β-alanyl NAD^+. (C) The NADPH-$K_3Fe(CN)_6$ reductase activity. The irradiated mixture contained 4.41×10^{-10} mol of complex I, 11.2×10^{-9} mol of arylazido-β-alanyl NAD^+ and NADH (○) [or NADPH (●)] at the indicated molar excess over the arylazido-β-alanyl NAD^+ concentration. The final volume was 0.18 mol. (D) As with (C) except that the irradiated mixture contained 24.2×10^{-9} mol of arylazido-β-alanyl-$NADP^+$ in place of arylazido-β-alanyl NAD^+.

photoactivation effect of this analogue. The finding that both arylazido pyridine nucleotide analogues photoactivate the NADPH-$K_3Fe(CN)_6$ reductase activity and that NADPH prevents only the photoactivation effect of arylazido-β-alanyl $NADP^+$ is an indication that the NADPH-$K_3Fe(CN)_6$ reductase may be distinct from the NADH-$K_3Fe(CN)_6$ reductase activity.

The Influence of Palmitoyl CoA on the Activation of NADPH-$K_3Fe(CN)_6$ Reductase following Photoirradiation in the Presence of Arylazido-β-alanyl $NADP^+$

Since the NADPH-$K_3Fe(CN)_6$ reductase activity of complex I is low, i.e., of the order of one-hundredth that of the NADH-$K_3(CN)_6$ activity, an active transhydrogenase utilizing or forming NADPH or an enzyme capable of reducing ferricyanide could conceivably interfere with the measurement of this activity. To test this possibility we have studied the effect of palmitoyl CoA on the NADPH-$K_3Fe(CN)_6$ reductase activity of complex I incubated with arylazido-β-alanyl NAD^+ or arylazido-β-alanyl $NADP^+$. Palmitoyl CoA is a known potent inhibitor of the NADPH-NAD^+ transhydrogenation reaction (Rydstrom *et al.*, 1971; Rydstrom, 1972). Palmitoyl CoA has been shown to protect specifically arylazido-β-alanyl $NADP^+$ (but not arylazido-β-alanyl NAD^+) labeling of the transhydrogenase peptide of complex I (Chen and Guillory, 1984). On the other hand, palmitoyl CoA does not influence the radioactive labeling of NADH dehydrogenase nor the NADH-NAD^+ transhydrogenase by either analogue (unpublished observations).

It can be seen from Table IV that there is a 30% inhibitory influence of 1.3 μM palmitoyl CoA on the measured NADPH-$K_3Fe(CN)_6$ reductase activity of complex I. There was no inhibition of NADH-$K_3Fe(CN)_6$ reductase at this concentration of palmitoyl CoA. In the presence of arylazido-β-alanyl NAD^+ the sensitivity of the NADPH-$K_3Fe(CN)_6$ reductase activity to palmitoyl CoA is increased (62.5% inhibition). This sensitivity to palmitoyl CoA is further increased following photoirradiation (81.8% inhibition). Thus the photodependent activation of the NADPH-

Table IV. The Effect of Palmitoyl-CoA on the NADPH-$K_3Fe(CN)_6$ Reductase Activity of Complex I in the Presence of Arylazido-β-alanyl NAD^+ or Arylazido-β-alanyl $NADP^+$

Additions	Percent activity	
	Dark	Photoirradiated
None	70	—
Arylazido-β-alanyl NAD^+	37.5	18.2
Arylazido-β-alanyl $NADP^+$	157.0	76.0

[a] Samples containing 0.98×10^{-10} mol of complex I were irradiated for 2 min at 5°C in a total volume of 0.2 ml and activities were assayed in the presence and absence of 1.3 mM palmitoyl-CoA. Activity in the absence of palmitoyl-CoA and pyridine nucleotide analogue (0.89 μmol $K_3Fe(CN)_6$ reduced min^{-1} mg $protein^{-1}$) was taken to represent 100% activity. When present arylazido-β-alanyl NAD^+ was at 3.47×10^{-9} mol and arylazido-β-alanyl $NADP^+$ at 24.2×10^{-9} mol.

$K_3Fe(CN)_6$ reductase observed in the presence of arylazido-β-alanyl NAD^+ is sensitive to palmitoyl CoA. This observation together with the known potent inhibitory action of the NAD^+ analogue on NADH-CoQ reductase would suggest that a major portion of the NADPH-$K_3Fe(CN)_6$ reductase activity present following treatment with the NAD^+ analogue represents transhydrogenase-driven ferricyanide reductase activity.

The NADPH-$K_3Fe(CN)_6$ reductase activity stimulated by arylazido-β-alanyl $NADP^+$ is much less sensitive to palmitoyl CoA than that stimulated by arylazido-β-alanyl NAD^+. The ferricyanide reductase activity, in the dark, is stimulated 57% by arylazido-β-alanyl $NADP^+$ in the presence of 1.3 μM palmitoyl CoA (Table IV), and following photoirradiation it is inhibited 24% by arylazido-β-alanyl $NADP^+$. Since arylazido-β-alanyl $NADP^+$ has been shown to be a potent inhibitor of transhydrogenation without influencing the other pryidine nucleotide-dependent reactions of complex I (Chen and Guillory, 1980), the activity measurable following photoirradiation in the presence of both palmitoyl CoA and arylazido-β-alanyl $NADP^+$ must be in large part due to oxidoreductase catalysis independent of transhydrogenation.

Effect of Arylazido-β-alanine on the NADH-$K_3Fe(CN)_6$ Reductase and NADH CoQ Reductase Activities of Complex I

In theory the pyridine nucleotide portion of the photoaffinity probes used in this study are the components which direct the photosensitive component, arylazido-β-alanine, to the vicinity of the nucleotide binding site. Upon light activation it is the latter which interacts in a presumably restricted way within the pyridine nucleotide binding region. If this were completely true, any binding or inhibitory effect due only to arylazido-β-alanine would be classified as being nonspecific.

We have observed (cf. Fig. 5) that the NADH-CoQ reductase activity of complex I is inhibited to a small but definite extent by arylazido-β-alanine following photoirradiation. At an inhibitor-to-complex I molar concentration ratio of 181 to 1, 40% inhibition of the NADH-CoQ reductase activity could be obtained. This can be contrasted with the strong inhibitory effect of arylazido-β-alanyl NAD^+ on this activity (K_i = 12 μM) which results in a 75% inhibition of NADH-CoQ reductase at a 25-fold excess of the NAD^+ analogue over the enzyme concentration (Chen and Guillory, 1979). Under identical conditions the NADH-$K_3Fe(CN)_6$ reductase was not influenced by arylazido-β-alanine (Fig. 5). This result would appear to indicate that arylazido-β-alanine has a definite relative specificity toward the two complex I activities. The photodependent inhibition of NADH-CoQ reductase by arylazido-β-alanine could not be prevented by the presence of either NADH

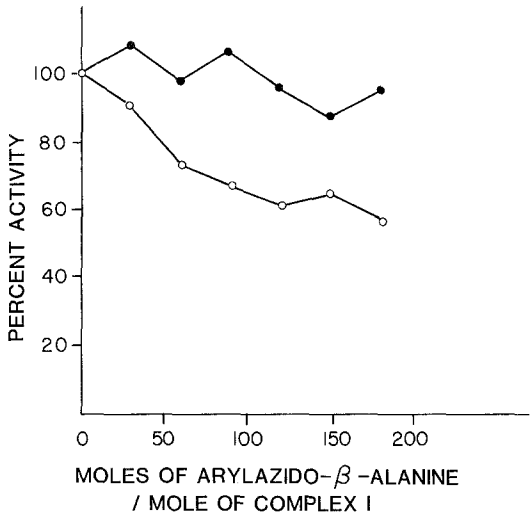

Fig. 5. The photodependent influence of arylazido-β-alanine on the NADH-CoQ_1 reductase and NADH-$K_3Fe(CN)_6$ reductase of complex I. The irradiation mixture (0.4 ml) contained 125 μM phosphate buffer, pH 7.0, 1.6×10^{-10} mol complex I, and arylazido-β-alanine at a molar excess over complex I as indicated in the legend to the figure. The activities were measured prior to and following photoirradiation, and the activity prior to photoirradiation was taken to represent 100% of activity. The NADH-CoQ_1 reductase (○) and NADH-$K_3Fe(CN)_6$ (●) reductase activities were assayed on 30 and 10 μl, respectively, of the irradiated mixtures.

or CoQ during photoirradiation. We interpret the lack of a protective influence of NADH or CoQ as indicating that the pyridine nucleotide and CoQ binding sites are not directly involved in the inhibitory activity of arylazido-β-alanine.

Discussion

Ferricyanide is an electron acceptor which can be utilized as a nonspecific acceptor for a number of different enzymatic activities (Minakami *et al.*, 1962). This report demonstrates that the NADH-$K_3Fe(CN)_6$ reductase activity of complex I is influenced by arylazido-β-alanyl NAD^+ and arylazido-β-$NADP^+$ in the dark or following photoirradiation, in a manner similar to that reported for NADH-CoQ

reductase (Chen and Guillory, 1979, 1980). However, in the case of the NADPH-$K_3Fe(CN)_6$ reductase the kinetic results are much more complicated. Both arylazido-β-alanyl NAD^+ and arylazido-β-alanyl $NADP^+$ were found to bring about a sizable activation of NADH-$K_3Fe(CN)_6$ reductase activity following photoirradiation. The likelihood that the mitochondrial transhydrogenase is capable of catalyzing a major ferricyanide reductase activity has not been considered up to now. However, the complex kinetic results obtained in studies of arylazido-β-alanyl NAD^+ and arylazido-β-alanyl NADP on NADPH-$K_3Fe(CN)_6$ reductase, the activation of the reductase by photoirradiation in the presence of both photoprobes, and the palmitoyl CoA sensitivity of the light-dependent photoprobe activation of the NADPH-$K_3Fe(CN)_6$ reductase activity can be adequately explained on this basis.

This possibility is considered even more likely when it is realized that the NADPH-$K_3Fe(CN)_6$ reductase activity of complex I is comparatively low (it is only one-hundredth of the NADH-$K_3Fe(CN)_6$ reductase activity). The $K_{m,app}$ (NADPH) for ferricyanide reductase, estimated at 1180 to 1250 μM (Tables I and II), is much greater than the concentration of NADPH (200 μM) used under the restricted conditions of the assay procedure. Consequently, during the course of the assay the pyridine nucleotide reactivity would be expected to be due to preferential interaction of nucleotide with the NADH dehydrogenase or NADPH transhydrogenase enzyme which possess stronger affinities for the pyridine nucleotides. Both of these sites may be capable of catalyzing the reduction of ferricyanide. Photoirradiation in the presence of arylazido-β-alanyl NAD^+ results in the inhibition of the oxidoreduction site but not that of ferricyanide reductase which is postulated as being associated with the NADPH-NAD^+ transhydrogenation. Considering the probability that both the oxidoreduction and transhydrogenase sites have similar k_m values for NADPH, a stimulation of the ferricyanide reductase activity catalyzed by the transhydrogenase enzyme would be expected as the result of the decreased availability of the stronger binding sites on the dehydrogenase.

The transhydrogenase activity of complex I photoirradiated in the presence of arylazido-β-alanyl $NADP^+$ would be expected to be inhibited while the oxidoreductase-driven ferricyanide reduction would be activated. The fact that NADPH is effective in protecting against the activating effect of arylazido-β-alanyl $NADP^+$ indicates that the arylazido-β-alanyl $NADP^+$ is reactive with an NADPH binding site, i.e., the site normally responsible for NADPH-NAD^+ transhydrogenation. The enhanced activity observed in the presence of NADH under these conditions is explained as being the result of the added nucleotide reacting as a substrate for the NADH-$K_3Fe(CN)_6$ reductase.

Palmitoyl CoA is a potent inhibitor of NADPH-NAD^+ transhydrogenation (Rydstrom *et al.*, 1971; Rydstrom, 1972). The increased sensitivity of the arylazido-β-alanyl NAD^+ photodependent activation of NADPH transhydrogenation to palmitoyl CoA in comparison to that activation stimulated by arylazido-β-alanyl $NADP^+$ (Table IV) suggests that the major activity present following treatment with arylazido-β-alanyl NAD^+ represents a transhydrogenation-driven ferricyanide reduction. The activation of NADPH-$K_3Fe(CN)_6$ reductase in the presence of palmitoyl CoA and arylazido-β-alanyl $NADP^+$ prior to photoirradiation indicates that this activity is primarily that due to the oxidoreductase-driven ferricyanide reduction. Following photoirradiation this activity was inhibited only to the extent of 24% by palmitoyl CoA, indicating again a low proportion of transhydrogenase-related ferricyanide reduction. Clearly the photodependent activation of the NADPH-$K_3Fe(CN)_6$ reductase by the NAD^+-arylazido analogue is much more sensitive to palmitoyl CoA than that activation due to the $NADP^+$ analogue. This is consistent with a major proportion of the overall reaction being due to a transhydrogenase-dependent reaction following inhibition by arylazido-β-alanyl NAD^+.

These results are thus consistent with the postulate that the NADH-$K_3Fe(CN)_6$ reduction involves principally the oxidoreductase enzyme while the complex I NADPH-$K_3Fe(CN)_6$ reduction represents a combination of the NADH dehydrogenase and the NADPH-NAD^+ transhydrogenation enzyme systems.

Influence of Arylazido-β-alanine on Complex I Reactions

Although the NADH-$K_3Fe(CN)_6$ reductase has been assumed to be a consequence of the oxidoreductase activities of complex I (Minakami *et al.*, 1962; Hatefi *et al.*, 1974; Singer, 1965; Huang and Pharo, 1971; Baugh and King, 1972), there are a number of experimental differences between these two activities. The NADH-CoQ reductase activity of complex I is sensitive to rotenone (Lingahl and Oberg, 1961), while the ferricyanide reductase activity of complex I is insensitive to this inhibitor. Coenzyme Q has been shown to react at a site following the rotenone-sensitive size (Gutman *et al.*, 1972), while $K_3Fe(CN)_6$ is believed to react at the NADH binding site or close to this region (Dookjewaard and Slater, 1976).

In our investigations of the influence of arylazido-β-alanine on the various reactions of complex I we have found that the NADH-CoQ reductase activity is inhibited by arylazido-β-alanine to a small but definite extent following photoirradiation. Under conditions in which the NADH-CoQ reductase activity was inhibited 40% the NADH-$K_3Fe(CN)_6$ reductase was not influenced by arylazido-β-alanine (Fig. 5). This result suggests that

arylazido-β-alanine has a certain specificity toward the enzymatic site responsible for NADH-CoQ reductase activity. The photodependent inhibition could not be prevented by the presence of either NADH or CoQ in the irradiation mixture, indicating that the alanine derivative was probably not reacting at either the NADH or the CoQ binding site. It may be that arylazido-β-alanine interacts at a hydrophobic area between the $K_3Fe(CN)_6$ and CoQ reactive site, perhaps in the vicinity of the rotenone binding site. An investigation of the inhibitory nature of arylazido-β-alanine on a more completely resolved preparation of NADH dehydrogenase may aid in our interpretation of the inhibitory site for this compound.

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