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NAD^+-INDUCED PHOSPHATE ACCEPTOR SPECIFICITY IN SUBMITOCHONDRIAL SYSTEMS

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SUMMARY

The nonspecific phosphorylation reaction in submitochondrial systems obtained by sonication became much more specific in the presence of pyridine nucleotides. Phosphorylation of IDP was inhibited by increasing concentrations of NAD^+, an inhibition which became biphasic upon the addition of hexokinase (EC 2.7.1.1), whereas phosphorylation of ADP was totally unaffected. The inhibition by NAD^+ was of a noncompetitive type with $K_i = 1.5 \cdot 10^{-4}$ M. The nicotinamide part of the molecule was immediately responsible for the inhibitory effect. Although rotenone or antimycin A interfered with the specificity promoted by NAD^+, the pyridine nucleotide effect was not due to the introduction of an energy-competing NAD^+ reduction. No evidence was found of an initiated selective nucleoside triphosphatase reaction, so the regulation was considered to take place at a primary event of the energy conservation mechanism and possibly exerted by an allosteric change.

It is suggested that endogenous pyridine nucleotides which are lost upon sonic disintegration of mitochondria may constitute a factor for regulation of the nucleoside specificity during oxidative phosphorylation.

INTRODUCTION

Submitochondrial particles obtained by sonic irradiation show limited specificity for the nucleoside part of di- and triphosphates involved in energy linked reactions[1]. As shown in the preceding article[2] the degree of specificity for nucleoside diphosphates as phosphate acceptors during oxidative phosphorylation in particulate systems varies with the substrate added. It is evident that for example IDP is phosphorylated to a much lower extent compared to ADP when NADH is the substrate, than when other substrates are used. A similarly low degree of efficiency can be demonstrated when ITP or GTP substitutes ATP as the energy source for succinate linked NAD^+ reduction[1]. The increased specificity may reflect inherent properties of the phosphorylation site in the NADH flavin region of the respiratory chain but since the endogenous mitochondrial pyridine nucleotides are lost upon sonic treatment a regulatory effect of these pyridine nucleotides upon nucleoside specificity could be a functional device worth investigating. Preliminary reports

Abbreviation: TMPD, *N,N,N',N'*-tetramethyl-*p*-phenylenediamine.

from this laboratory[3] have supplied data which support this latter assumption and have shown oxidized NAD^+ to be a most powerful agent to increase the nucleoside specificity. Experiments where NAD^+ have been substituted by analogues changed either in the nicotinamide or in the adenine part of the pyridine nucleotide molecule indicate the nicotinamide part to be responsible for the specificity induction effect.

MATERIALS

All nucleotides, alcohol dehydrogenase (EC 1.1.1.1) and antimycin A were purchased from Sigma Chemical Co. (St Louis, Mo., U.S.A.). ^{32}P was obtained from the Radiochemical Centre (Amersham, U.K.). Hexokinase (EC 2.7.1.1) was bought from Boehringer and Soehne GmbH (Mannheim, Germany) and *N,N,N',N'*-tetramethyl-*p*-phenylenediamine (TMPD) from Eastman Organic Chemicals (Rochester, N.Y. U.S.A.). All other chemicals were of reagent grade.

METHODS

Submitochondrial particles of beef heart mitochondria were prepared according to LÖW AND VALLIN[4] with the minor modifications introduced by VALLIN[5]. Protein content was determined according to GORNALL *et al.*[6]. Oxygen consumption was measured polarographically with a Clark oxygen electrode (Yellow Springs Instrumental Co. Inc. No. 4004) in a thermostable electrode chamber at 30° in a basic medium containing 50 mM glycylglycine buffer (pH 7.5), 6 mM $MgCl_2$, 0.15 M sucrose, 3 mM phosphate buffer (pH 7.5), 2 mM nucleoside diphosphate as indicated, 20 mM glucose, hexokinase in excess and 0.3 mg of submitochondrial particle protein per ml. 3 mM sodium succinate or 5 mM sodium ascorbate *plus* 0.3 mM TMPD was added as substrate giving a final volume of 1.0 ml. Phosphate esterification was determined by the ^{32}P distribution method described by LINDBERG AND ERNSTER[7].

The reduction of NAD^+ (and analogues) was studied by the change of absorbance at given wavelengths in a Hitachi–Perkin–Elmer 124 spectrophotometer. Submitochondrial particles (0.15 mg protein per ml) were preincubated for 6 min in a medium containing 50 mM Tris–HCl (pH 8.0), 6 mM $MgCl_2$, 0.25 M sucrose, 2 mM KCN and 1 mM nucleosidetriphosphate as indicated. The reaction was started by addition of 10 mM sodium succinate and 1.5 mM NAD^+ (or analogue) giving a final volume of 3 ml. A homogeneous distribution of the reagents in the cuvette was attained by bubbling nitrogen through the reaction mixture for a short period of time and the temperature in the cuvette chamber was maintained at 30°.

The liberation of phosphate from nucleosidetriphosphates was studied in a system equivalent to that used for oxidative phosphorylation except that phosphate, glucose and hexokinase were excluded and 2 mM ATP or ITP was used instead of the nucleoside diphosphates. Determination of P_i liberated was made according to LINDBERG AND ERNSTER[7].

RESULTS

Effects of pyridine nucleotides on IDP phosphorylation

The effect of increasing concentrations of NAD^+ when added to a submitochondrial particulate system with succinate as the substrate and ADP or IDP as the

phosphate acceptor is demonstrated in Fig. 1. The efficiency of phosphorylating IDP was markedly decreased in the presence of the pyridine nucleotide and the further addition of hexokinase *plus* glucose seemed to change the inhibition from a linear into a biphasic course. The same pattern can be demonstrated with GDP as the phosphate acceptor whereas phosphorylation of ADP was completely unaffected by NAD^+ additions both in the presence and absence of a hexokinase trap. The decrease in phosphorylative capacity obtained by NAD^+ was not followed by a simultaneous reduction of the pyridine nucleotide as checked fluorimetrically. Although the phosphorylation of IDP was sensitive to increasing concentrations of NAD^+ the oxygen consumption remained constant over the whole concentration range tested. Addition of NAD^+ gave no further change of the pH-dependence found for phosphorylation of IDP[2].

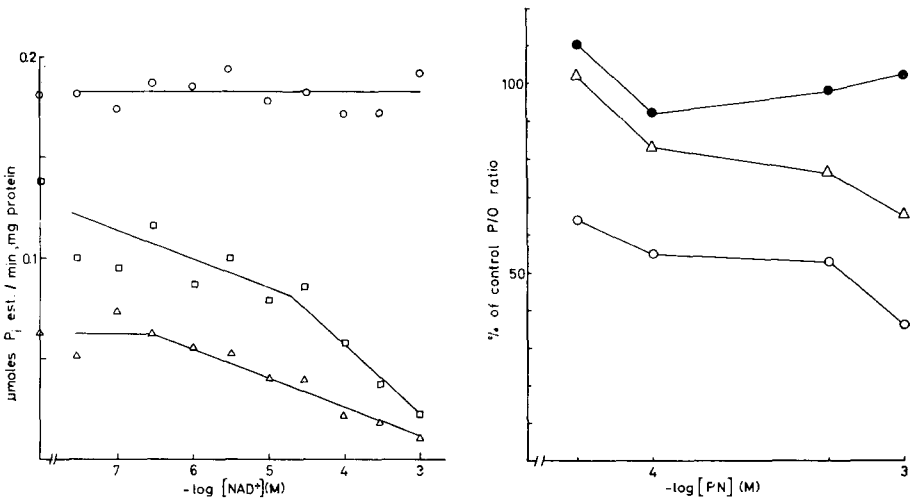

Fig. 1. The effect of NAD^+ on oxidative phosphorylation with succinate as the substrate. The electrode chamber contained in a final volume of 1.0 ml, 50 mM glycylglycine buffer (pH 7.5), 0.15 M sucrose, 6 mM $MgCl_2$, 3 mM phosphate buffer (pH 7.5), 2 mM ADP or IDP, 0.3 mg of submitochondrial particle protein, NAD^+ as indicated. 20 mM glucose and hexokinase in excess were added when indicated. The reaction was started by adding 3 mM succinate and the temperature was 30°. ○—○, ADP; △—△, IDP; □—□, IDP + glucose and hexokinase.

Fig. 2. The effect of rotenone on pyridine nucleotide-induced decrease of IDP phosphorylation with succinate as the substrate. Experimental conditions as in Fig. 1 including glucose and hexokinase. 0.4 nmoles of rotenone per mg protein and pyridine nucleotide (PN) concentration as indicated, were used. ○—○, NAD^+; ●—●, NAD^+ + rotenone; △—△, NADH + rotenone.

NADPH had no effect on P/O ratios with IDP or GDP as the phosphate acceptor and $NADP^+$ was about half as effective as NAD^+ of the same concentration to diminish these P/O ratios. This type of particles are unable to reduce $NADP^+$ without the further addition of NADH to complete a transhydrogenase system[8].

Effects of rotenone and antimycin A

Fig. 2 demonstrates that the effect of NAD^+ on P/O ratios obtained with IDP as phosphate acceptor could be essentially counteracted if rotenone was added

to a system where succinate was the substrate. Rotenone was, however, less effective if NAD^+ was replaced by NADH.

A similar and even more pronounced decrease in phosphorylative activity as well as P/O ratio could be demonstrated upon the addition of NAD^+ to particles phosphorylating IDP with ascorbate *plus* TMPD as the substrate (Fig. 3). As seen from the figure, this effect of NAD^+ was almost completely abolished by a further addition of antimycin A, whereas the addition of rotenone was less effective. A comparison with Fig. 2 indicates that the rotenone-induced inhibition was less complete if TMPD *plus* ascorbate was used instead of succinate.

Further investigation of the rotenone effect by titration, Fig. 4, revealed that the gradual return of the capacity to phosphorylate IDP followed a sigmoidal pattern.

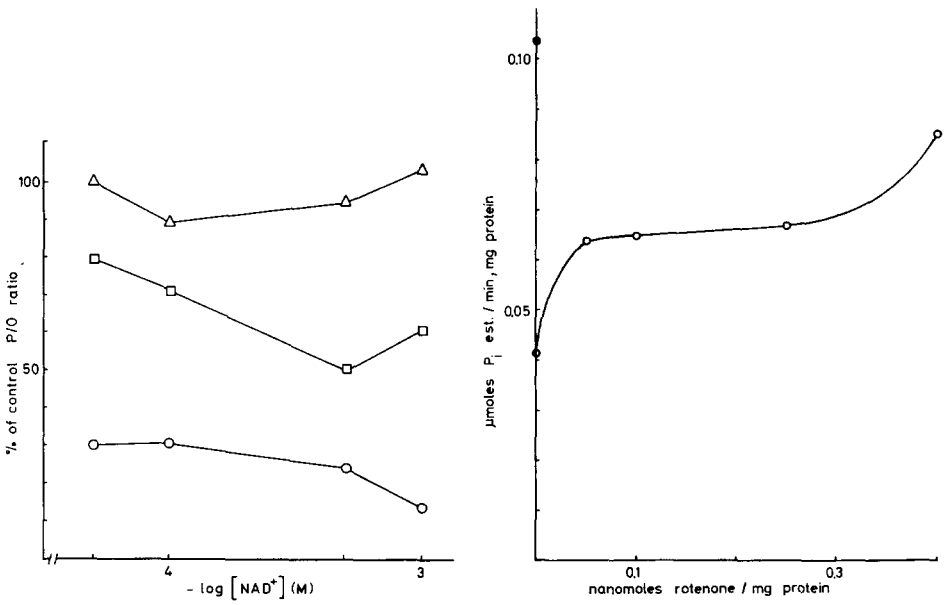

Fig. 3. The effect of rotenone and antimycin A on NAD^+-induced decrease of IDP phosphorylation with ascorbate *plus* TMPD as the substrate. Experimental conditions as in Fig. 2, but with 5 mM ascorbate *plus* 0.3 mM TMPD instead of succinate. The rotenone concentration was 0.4 nmole/mg protein and the antimycin A concentration 0.3 µg/mg protein. ○—○, without inhibitor; □—□, + rotenone; △—△, + antimycin A.

Fig. 4. The effect of rotenone on NAD^+-induced decrease of IDP phosphorylation with succinate as the substrate. Experimental conditions as in Fig. 1 with the following modifications: the NAD^+ concentration was $5 \cdot 10^{-4}$ M; 20 mM glucose and hexokinase in excess was added to the medium. ● represents the control without NAD^+ present.

Structural background of the NAD^+ effect

Various analogues of NAD^+ were tested for their regulatory effects (Table I). Analogues with substitution in the nicotinamide part of the molecule had a weaker repressive effect on phosphorylation of IDP. This effect was, however, retained in analogues structurally changed in the adenine part of the molecule. If the nicotinamide part of the NAD^+ molecule thus was mainly responsible for the induction of

nucleoside specificity, nicotinamide or nicotinamide mononucleotide could not substitute the entire pyridine nucleotide.

For comparison, the efficiency of the various NAD^+ analogues as electron acceptors in a succinate linked reversal of electron transfer with ITP or ATP as the energy source is demonstrated in Table II. Analogues with a high capacity to interfere with the synthesis of ITP (Table I) did not turn out as good electron acceptors (Table II) and thus there was no obvious correlation between these two functions.

TABLE I

THE EFFECT OF NAD^+ ANALOGUES ON IDP PHOSPHORYLATION WITH SUCCINATE AS THE SUBSTRATE

Experimental conditions as in Fig. 1, including glucose and hexokinase.

Dinucleotide added ($5 \cdot 10^{-4}$ *M*)	*Respiration* (*μatoms O consumed per min per mg protein*)	*Phosphorylation* (*μmoles P_i esterified per min per mg protein*)	*P/O*
—	0.188	0.107	0.57
NAD^+	0.196	0.047	0.24
3-AAD^+	0.154	0.060	0.39
3-PAD^+	0.161	0.057	0.35
NHD^+	0.220	0.043	0.20
3-AHD^+	0.175	0.031	0.18
3-PHD^+	0.182	0.055	0.30
$TNAD^+$	0.186	0.058	0.31

Abbreviations: 3-AAD^+, 3-acetylpyridine-adenine dinucleotide; 3-PAD^+, 3-pyridinealdehyde-adenine dinucleotide; NHD^+, nicotinamide-hypoxanthine dinucleotide; 3-AHD^+, 3-acetylpyridine-hypoxanthine dinucleotide; 3-PHD^+, 3-pyridinealdehyde-hypoxanthine dinucleotide; $TNAD^+$, thionicotinamide-adenine dinucleotide.

TABLE II

REDUCTION OF NAD^+ AND SOME ANALOGUES BY SUCCINATE

Submitochondrial particles, 0.15 mg protein per ml, were preincubated for 6 min (30°) in a medium containing 50 mM Tris–HCl (pH 8.0), 6 mM $MgCl_2$, 0.25 M sucrose, 2 mM KCN and 1 mM ATP or ITP. The reaction was started by addition of 1.5 mM pyridine nucleotide and 10 mM succinate. Final volume 3 ml. The spectral properties of the analogues from Pabst Laboratories, Milwaukee, Wisc., U.S.A., circular OR-18 (1961).

Pyridine nucleotide	*Absorption change at wavelength* (*nm*)	*Extinction coefficient* (*mM*)	*Acceptor reduced* (*nmoles/min per mg protein*)	
			With ATP	*With ITP*
NAD^+	338	6.2	47	13
3-AAD^+	363	9.1	53	19
3-PAD^+	358	9.3	42	12
NHD^+	338	6.2	76	9
3-AHD^+	361	9.0	64	15
3-PHD^+	356	9.4	32	12
$TNAD^+$	395	11.3	36	7

Abbreviations: see Table I.

NAD^+ reduction or ITPase?

The experiments described so far could indicate that addition of NAD^+ introduced conditions for an energy-dependent reversal of electron transfer which would compete for the high energy generated in the case where the affinity between nucleoside triphosphates like ITP or GTP and hexokinase is less pronounced. The effect of rotenone and antimycin A would then be an inhibition of the reversed electron transfer reaction. If there were a delicate balance between two such energy competing systems, a change in equilibrium would follow from a moderate variation in the amount of hexokinase added. The results obtained were, however, reverse to what then would be expected, a decrease in hexokinase concentration gave more IDP phosphorylated, whereas more hexokinase added tended to diminish the phosphorylative activity in the presence of NAD^+. The effect of NAD^+ could possibly be visualized as an initiation of a preferential hydrolysis of the ITP formed. The data reported so far were obtained almost exclusively in the presence of hexokinase, but the proposed effect would be more accentuated if the trap was omitted from the system. An experiment where high initial concentrations of nucleoside triphosphates were added under otherwise identical conditions is shown in Table III. The quotient between ITP/ATP hydrolyzed remained essentially unchanged except upon the addition of NAD^+ in combination with ascorbate *plus* TMPD, where splitting of ATP was the more stimulated activity.

TABLE III

ATPase AND ITPase IN AN AEROBIC SYSTEM

The medium contained 50 mM glycylglycine buffer (pH 7.5), 6 mM $MgCl_2$, 0.15 mM sucrose, 2 mM ATP or ITP and 0.3 mg of submitochondrial particle protein per ml. Additions where indicated were 5 mM sodium succinate, 10^{-3} M NAD^+, 5 mM sodium ascorbate and 0.3 mM TMPD. The final volume was 1 ml and the temperature was 30°. Determination of P_i liberated during 5 min was made colorimetrically according to LINDBERG AND ERNSTER[7].

Additions	*Amounts of P_i released (μmoles P_i per min per mg protein)*		*ITPase/ATPase*
	ATP	*ITP*	
None	0.374	0.250	0.67
Succinate	0.385	0.261	0.68
Succinate, NAD^+	0.365	0.266	0.73
NAD^+	0.371	0.254	0.68
Ascorbate, TMPD	0.437	0.309	0.71
Ascorbate, TMPD, NAD^+	0.619	0.360	0.58

Type of inhibition

A kinetic study of the NAD^+ effect on phosphorylation of IDP is demonstrated in Fig. 5. From the Lineweaver–Burk plot emerges that the inhibitory effect was of a noncompetitive type and additional experiments gave a K_i value for NAD^+ of $1.5 \cdot 10^{-4}$ M.

These experiments indicate that the regulatory influence of NAD^+ is exerted on a level which probably is separate from the final phosphorylative event.

DISCUSSION

We have demonstrated earlier[9] that the energy-dependent reduction of NAD^+ can compete with the synthesis of ATP for part of the energy generated. A similar competition between energy-linked transhydrogenase reaction and oxidative phosphorylation was investigated in the same type of particles by LEE AND ERNSTER[10].

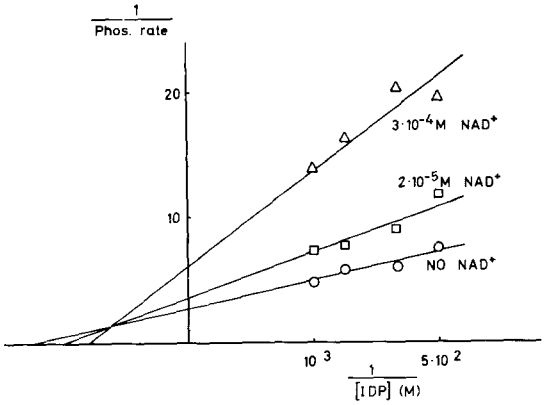

Fig. 5. Lineweaver–Burk plot of the rate of phosphate esterification *versus* the concentration of IDP. Experimental conditions as in Fig. 1 without glucose and hexokinase and with various concentrations of IDP and NAD^+ as indicated. The phosphorylative rate is expressed as μmoles P_i esterified per min and per mg protein.

From Figs. 1–3 it is evident, however, that the addition of NAD^+ to particles oxidizing succinate or ascorbate *plus* TMPD does not cause a decrease in phosphorylative activity or P/O ratio if ADP is the phosphate acceptor even if hexokinase is omitted from the system. The incomplete respiratory control in these particles[5] seems to cause conditions where the level of primary high energy intermediates is insufficient to counteract the electron flow in an uninhibited respiratory chain as in the present systems. The energy-competing systems mentioned above are both characterized by two separate sources of reducing equivalents and the presence of a respiratory inhibitor. A number of evidences favour the conclusion that the decrease in IDP phosphorylation caused by NAD^+ is not due to the introduction of an energy competing NAD^+ reduction:

(a) No reduction of the NAD^+ added could be detected fluorimetrically even in highly sensitive measurements.

(b) The decrease in IDP phosphorylation was not accompanied by a simultaneous decrease in respiratory activity.

(c) Rotenone could substitute antimycin A only partially when counteracting the influence of NAD^+ on IDP phosphorylation with ascorbate *plus* TMPD as the substrate.

(d) Variations in hexokinase concentration gave no indication of two energy-competing systems in equilibrium.

(e) No correlation between ability of various NAD^+ analogous to suppress IDP phosphorylation and to act as electron acceptor in a reversed electron transfer system.

(f) NADH depressed the IDP phosphorylation activity in the presence of a complete rotenone block.

We have been unable to correlate the decrease in phosphorylative capacity caused by NAD^+ with a simultaneous stimulation of the ITPase. The inhibition was further noncompetitive and thus a direct interaction of NAD^+ on the binding site for inosine nucleotides on the phosphorylation enzyme was less likely. The demonstrated effect of respiratory inhibitors like rotenone and antimycin A, *plus* the fact that the ratio between IDP and ADP phosphorylated varied with the different substrates added and the number of energy conservation sites involved[2] would sooner suggest that the specificity of the phosphorylation process is regulated prior to the ultimate phosphorylation reaction.

From the data presented it is evident that the nicotinamide part of the pyridine nucleotide plays the immediate rôle when phosphorylation of IDP is affected but also the size of the molecule is of certain importance.

It is tempting to suggest that the pyridine nucleotide interacts directly with respiratory chain components preceding the energy conservation sites involved possibly by causing conformational changes. Rotenone or antimycin A, respectively, would then restore these enzymatic changes. The ability to select between the different nucleoside parts may also imply that an early involvement of IDP or ADP in the energy conservation sequence before the ultimate phosphorylation event has to be taken into consideration.

Endogenous pyridine nucleotides which are lost upon sonic disintegration of mitochondria seem thus to have a regulatory effect on the specificity of the primary phosphorylation event. The data suggest that allosteric regulation of primary processes in oxidative phosphorylation may play a rôle hitherto unobserved in intact mitochondrial systems.

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REFERENCES

1 H. LÖW, I. VALLIN AND B. ALM, in B. CHANCE, *Energy-Linked Functions of Mitochondria*, Academic Press, New York, 1963, p. 5.
2 I. VALLIN AND P. LUNDBERG, *Biochim. Biophys. Acta*, 256 (1971) 179.
3 I. VALLIN, P. LUNDBERG AND H. LÖW, *Biochem. Biophys. Res. Commun.*, 36 (1969) 519.
4 H. LÖW AND I. VALLIN, *Biochim. Biophys. Acta*, 69 (1963) 361.
5 I. VALLIN, *Biochim. Biophys. Acta*, 162 (1968) 477.
6 A. G. GORNALL, C. J. BARDAWILL AND M. M. DAVID, *J. Biol. Chem.*, 177 (1949) 751.
7 O. LINDBERG AND L. ERNSTER, in D. GLICK, *Methods of Biological Analysis*, Vol. 3, Interscience, New York, 1956, p. 1.
8 L. DANIELSON AND L. ERNSTER, in B. CHANCE, *Energy-Linked Functions of Mitochondria*, Academic Press, New York, 1963, p. 157.
9 I. VALLIN AND H. LÖW, *Biochim. Biophys. Acta*, 92 (1964) 446.
10 C. P. LEE AND L. ERNSTER, *Eur. J. Biochem.*, 3 (1968) 385.