STUDIES ON THE MECHANISM OF OXIDATIVE PHOSPHORYLATION

II. ROLE OF BOUND PYRIDINE NUCLEOTIDE IN PHOSPHORYLATION

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In previous communications[1,2] we have described the "separation" of beef heart mitochondrial suspensions into a difficultly sedimentable phosphorylating electron transport particle and a heavier particle which has the characteristics of a modified mitochondrion. Both particles carry on oxidative phosphorylation with P/O ratios close to the highest recorded in the literature. The light particle has been called "PETP"** while the heavy particle is referred to simply as the "residue" particle. The present communication deals with (1) the requirement of pyridine nucleotide for maximal oxidation of various substrates by PETP and residue; (2) the dependence of the phosphorylation on the state of pyridine nucleotide *i.e.* whether free or bound; and (3) the conditions under which external pyridine nucleotide can be reincorporated into the residue particles.

In 1950 HUENNEKENS AND GREEN[3,4] reported the presence in mitochondria of considerable amounts of the bound forms of DPN, TPN, cocarboxylase and the adenine nucleotides. They presented evidence that the bound pyridine nucleotides were released from mitochondria under a variety of experimental conditions such as prolonged storage at 0°, exposure to deionized water, arsenite and organic solvents. Subsequently HUNTER *et al.*[5,6] examined the relation between the state of the mitochondrion and the level of bound pyridine nucleotide while JACOBSON[7] has reported that bound mitochondrial DPN is not acted upon by *Neurospora* DPNase.

RESULTS

Methods and materials

All other pertinent information about methods of determination, preparation of particles, source of materials, cofactors, etc. has been provided in the previous communications[1,2]. Twice recrystallized alcohol dehydrogenase was purchased from Worthington Biochemical Corp. Pyruvate and α-ketoglutarate were determined according to FRIEDEMANN AND HAUGEN[8].

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** The following abbreviations will be used in the text: electron transport particle, ETP; phosphorylating electron transport particle, PETP; diphosphopyridine nucleotide, DPN; reduced diphosphopyridine nucleotide, DPNH; triphosphopyridine nucleotide, TPN; adenosine triphosphate, ATP; coenzyme A, CoA; alcohol dehydrogenase, ADH.

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Effect of cofactors on oxidation and phosphorylation

The rate of oxidation of substrates of the following pyridinoprotein enzymes — α-ketoglutarate, β-hydroxybutyrate, malate + pyruvate and glutamate — is increased to a greater or lesser degree by addition of DPN and other cofactors (*cf.* Table II of the previous communication by ZIEGLER *et al.*[1]). The requirement shows up best with the residue particles and least with the mitochondrial suspension. The increase in rate of oxidation induced by addition of cofactors is not necessarily the same for all substrates oxidized by a given particle suspension. Thus the oxidation induced by α-ketoglutarate usually responds to a lesser degree than does that induced by β-hydroxybutyrate.

The extra oxidation induced by addition of cofactors (cofactor-induced oxidation) is accompanied by essentially normal phosphorylation in the case of mitochondria and residue particles but by little or no phosphorylation in the case of PETP. Only the oxidations catalyzed by PETP which do not depend upon added cofactors involve normal phosphorylation. Thus the mitochondrion and the residue particle possess the capacity to utilize external DPN (and other cofactors) in the form essential for oxidative phosphorylation whereas PETP has lost this capacity as a consequence of the procedure by which it is extracted from mitochondria.

Coenzyme A and cocarboxylase are specifically required only for the oxidation of α-ketoglutarate[9,10] and pyruvate[9,11] whereas DPN is required for all the pyridinoprotein enzymes except for the *iso*citric dehydrogenase which is specific for TPN[12].

There is a time factor in the emergence of a requirement for cofactors. Fresh mitochondria show little or no requirement whereas aged mitochondria show progressively greater requirement. This applies even more so to the residue particle (*cf.* Table I). However, PETP does not show much change in the degree of response of its pyridinoprotein enzymes to the addition of cofactors upon aging.

Restoration of phosphorylation capacity by exposure to cofactors

When suspensions of aged residue particles are washed in 0.25 *M* sucrose the oxidative activity which they exhibit in absence of added cofactors is reduced sharply (Table I).

TABLE I

EFFECT OF AGING ON THE RESPONSE OF THE RESIDUE PARTICLES TO WASHING

	Activity of fresh suspensions after washing					*Activity of aged suspensions after washing*			
	*—cofactors**		*+cofactors*			*—cofactors*		*+cofactors*	
	*O***	*P****	*O*	*P*		*O*	*P*	*O*	*P*
A	0.059	0.089	0.068	0.150	A′	0.022	0.028	0.061	0.081
B	0.036	0.048	0.115	0.139	B′	0.015	0.031	0.099	0.117
C	0.038	0.090	0.058	0.076	C′	0.005	0.004	0.047	0.083

* The cofactor mixture contained 1 μmole DPN, 0.5 μmole cocarboxylase and 0.1 μmole CoASH. The mixture was added to each manometer vessel (final vol. 3 ml).

** μatoms oxygen per min per mg protein in presence of pyruvate + malate.

*** μmoles phosphate esterified per min per mg protein in presence of pyruvate + malate.

A – Residue of a PETP preparation; A′ – As A only stored at —15° for 4 months, then washed three times with 0.25 *M* sucrose.

B – Residue of an ETP preparation[13]; B′ – As B only stored at —15° for 1 month, then washed twice with 0.25 *M* sucrose.

C – Residue of a PETP preparation; C′ – As C only stored at 15° for 4 months, treated with alkali to pH 8.5, then washed three times with 0.25 *M* sucrose.

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However, in most cases the P/O ratio is only slightly affected. The sensitivity of the particles to washing is a function of the age of the material. Freshly prepared residue particles are only slightly affected by washing, whereas suspensions stored at —15° for several months are almost completely inactivated after several washes. After exposure of the particles to alkali (pH 8.5) the process of aging is accelerated as attested by the efficiency of the washing procedure. When tested in presence of added cofactors the rates of oxidation and phosphorylation of the washed suspensions approach those obtained prior to the washing procedure.

If the particle suspension is incubated at 0° for 10 minutes with various cofactors, substrate, magnesium ions and Versene EDTA at the following final concentrations per ml: 3 μmoles $MgCl_2$, 3 μmoles Versene, 6 μmoles pyruvate, 2 μmoles malate, 0.3 μmole DPN, 0.3 μmole TPN, 0.15 μmole cocarboxylase, 0.03 μmole CoA, and 3 μmoles glutathione, then the particles so exposed do not show the sharp decline in oxidative rate which follows extensive washing in 0.25 *M* sucrose. Under the same conditions of assay the rate of oxidation is, if anything, higher following the exposure to cofactors. Furthermore the rate of phosphorylation is not increased by addition of cofactors in the assay system although the rate of oxidation is usually stimulated (Table II).

TABLE II

EFFECT OF PREINCUBATION ON THE RESPONSE OF AGED RESIDUE PARTICLES TO WASHING

Conditions	*Assay conditions*			
	—cofactors		*+cofactors**	
	*O***	*P****	*O***	*P****
Residue§ not washed after storing	0.034	0.078	0.085	0.148
Residue washed 3 times with 0.25 *M* sucrose	0.009	0.015	0.094	0.142
Residue preincubated then washed 3 times	0.051	0.144	0.093	0.146

* For details of cofactor mixture see legend to Table I.

** μatoms oxygen per min per mg protein in presence of pyruvate + malate.

*** μmoles phosphate esterified per min per mg protein.

§ The residue used in this experiment had been treated at pH 8.5, washed in 0.25 *M* sucrose and then stored at —10° for 1 week.

This type of experiment suggests that the residue particles as prepared catalyze various oxidation processes requiring pyridine nucleotide with or without accompanying phosphorylation. The oxidation which involves the bound pyridine nucleotide is invariably associated with phosphorylation while only a fixed moiety of the oxidation involving external pyridine nucleotide can be coupled. This upper limit presumably is set by some component or characteristic of the system as yet unknown. As a consequence of the preincubation with cofactors, the residue particles phosphorylate maximally and show the full oxidative activity which normally accompanies this phosphorylation when tested in absence of added cofactors. The cofactor-induced oxidation now is unaccompanied by phosphorylation.

PETP rarely shows this type of behavior and even so not to anything like the same degree. The washing procedure does not reduce the oxidative rate in absence of added cofactor very significantly and the preliminary incubation with cofactors does not lead to a marked increase in the oxidative rate without cofactors.

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Some studies have been conducted which were directed towards pinpointing those components of the incubation mixture which are essential for the restoration of oxidative and phosphorylative activity of the residue particles. Substrate, magnesium ions and versenate without added cofactors have a partial restorative effect (*cf.* Table III). Similarly cofactors without substrate, magnesium ions and versenate are ineffective. DPN is the key coenzyme since its omission abolishes the restorative effect of the cofactor mixture. In fact in some experiments DPN alone was virtually equivalent to the cofactor mixture. Although the P/O ratios reported in Table III are considerably lower than the maximal values attainable, the qualitative nature of the effects is the same whether the P/O ratio is high or low. These data were chosen because the various determinations were carried out with the same preparation at one time.

TABLE III

COMPONENT STUDY OF THE FORTIFIED SUCROSE MEDIUM WITH WHICH THE RESIDUE PARTICLES ARE PREINCUBATED BEFORE THE WASHING PROCEDURE

	Components omitted from preincubation medium	*Rate of oxidation μatoms oxygen/min/mg*	*Rate of phosphorylation μmoles P/min/mg*
1	None	0.052	0.056
2	All	0.008	0.012
3	DPN, Versene, substrate, Mg^{++}	0.012	0.014
4	Cocarboxylase, TPN	0.041	0.031
5	Versene, Mg^{++}	0.013	0.015
6	Substrate	0.011	0.011
7	DPN, substrate	0.012	0.018

The complete system contained $MgCl_2$, Versene, pyruvate + malate, DPN, cocarboxylase, CoA and glutathione at the concentrations designated in the text.

The particles preincubated under the conditions specified above were then washed exhaustively in 0.25 *M* sucrose. Oxidation and phosphorylation were measured in the presence of pyruvate + malate as substrates.

Phosphorylation during DPNH oxidation

Although PETP and mitochondria catalyze the rapid oxidation of DPNH, there is no concomitant phosphorylation (Table IV). To decide whether external DPNH has an uncoupling action in this system, DPNH and a citric acid cycle substrate were oxidized simultaneously and the phosphorylation measured. The presence of DPNH added as such or generated by the alcohol dehydrogenase system inhibited substrate oxidation (pyruvate + malate more than α-ketoglutarate). However, whatever oxidation did take place led to substantial phosphorylation. Hence, an uncoupling role cannot be assigned to DPNH.

Reduction of DPN by various substrates

The above results demonstrate a qualitative difference between external DPNH and DPNH generated within the bound pyridinoprotein complex which is bonded to the heme chain. It was therefore of considerable interest to determine whether external DPN was reducible by any of the substrates of pyridinoprotein enzymes in PETP (with appropriate supplementation of necessary coenzymes). No measurable reduction was observed in presence of α-ketoglutarate, pyruvate + malate and glutamate. When

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TABLE IV

PHOSPHORYLATION ACCOMPANYING OXIDATION OF DPNH BY MITOCHONDRIA AND PETP IN ABSENCE AND PRESENCE OF CITRIC ACID CYCLE SUBSTRATES

Expt.	*Preparation*	*Substrate*	*ΔO* μatoms	*ΔSubstrate* μmoles	*ΔP* μmoles	*P/O*
1	PETP (8.4 mg)	DPNH	7.0	—	0.0	0.0
		Pyruvate + malate	6.2	—	12.7	2.05
		DPNH + pyruvate + malate	11.7	—	2.6	0.55*
2	Mitochondria (4.7 mg)	Pyruvate + malate	13.2	2.9	23.6	1.79
		Alcohol dehydrogenase (ADH) + ethanol	4.3	—	0.0	0.0
		Pyruvate + malate + ADH + ethanol	11.6	1.9	11.9	1.63*
3	Mitochondria (6.2 mg)	α-Ketoglutarate	5.4	3.6	9.3	1.72
		ADH + ethanol	7.0	—	0.0	0.0
		α-Ketoglutarate + ADH + ethanol	11.0	2.9	8.2	2.05*

Each flask contained 1 μmole DPN (in the ADH experiments) and where indicated 150 γ ADH 100 μmoles ethanol, 10 μmoles DPNH. Additions concerned with oxidative phosphorylation measurements as previously indicated.

* P/O ratio corrected for oxygen uptake due to DPNH oxidation; *e.g.*, in Experiment no. 2: P/O = (11.9/11.6—4.3) = 1.63.

α-ketoglutaric dehydrogenase was added to the same incubation mixture the DPN was immediately reduced. However reduction of DPN was readily demonstrable with β-hydroxybutyrate as substrate. Similarly TPN (but not DPN) was readily reducible in presence of *iso*citrate as substrate. It seems therefore that the dehydrogenase system which show the highest phosphorylation (α-ketoglutaric, pyruvic, malic and glutamic) do not reduce external DPN whereas the less efficiently coupled systems do so at least in part.

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SUMMARY

The citric cycle oxidations of PETP which are induced by external DPN are not coupled to phosphorylation whereas the oxidation involving bound pyridine nucleotide is coupled. The residue particles differ from PETP in that a proportion of the DPN-induced oxidation is still coupled. With extensive washing of the residue particles the capacity to catalyze oxidation in absence of added cofactors is sharply reduced. This capacity can be restored by incubating the particles with cofactors and substrate at 0° and then washing them exhaustively. Such treated particles regain both their original oxidative and phosphorylative capacity (in absence of added cofactors). DPNH oxidation is not accompanied by phosphorylation. External DPN is not reducible by the substrates of tightly coupled pyridinoprotein dehydrogenase systems.

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ACTION DE L'ACTINOMYCETINE SUR LES PAROIS CELLULAIRES BACTERIENNES

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Depuis la mise au point de techniques adéquates d'isolement des parois cellulaires microbiennes, nos connaissances sur leur constitution physique et chimique se sont précisées et ont été récemment revues et discutées par l'un de nous[1,2].

L'étude de la structure macromoléculaire des parois cellulaires échappe évidemment à l'analyse chimique. Un des moyens d'investigation réside dans la caractérisation des fragments obtenus après digestion enzymatique. L'obtention d'enzymes actifs sur les parois cellulaires constitue donc un outil de recherche précieux et l'intérêt présenté par de telles substances deviendra éminent le jour où la paroi cellulaire d'un microorganisme déterminé pourra être digérée par plusieurs enzymes s'attaquant à des types de liaison spécifiquement différents.

Le lysozyme, découvert en 1922 par FLEMING[3], dont le substrat type est représenté par les parois de *Micrococcus lysodeikticus*, et l'enzyme "streptolytique" isolé par MCCARTY en 1952[4] sont, à l'heure actuelle, les seuls enzymes bactériolytiques disponibles dans un état suffisamment pur pour autoriser une étude significative des produits de dégradation obtenus après action sur les parois cellulaires. L'un et l'autre de ces enzymes s'attaquent à un substrat de même nature générale mucoïde. Ils en libèrent des substances réductrices et sont certainement indifférents aux liaisons peptidiques qui forment certains chaînons structurels des parois cellulaires.

LIESKE, en 1921[5], et GRATIA ET DATH, en 1924[6], mettaient indépendamment

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