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TIGHTLY BOUND NUCLEOTIDES OF THE ENERGY-TRANSDUCING ATPase, AND THEIR ROLE IN OXIDATIVE PHOSPHORYLATION

I. THE *PARACOCCUS DENITRIFICANS* SYSTEM

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SUMMARY

1. The coupling ATPase of *Paracoccus denitrificans* can be removed from the membrane by washing coupled membrane fragments at low salt concentrations.

2. This ATPase resembles coupling ATPases of mitochondria, chloroplasts and other bacteria. It is a negatively charged protein of molecular weight about 300 000. An inhibitor protein is bound tightly to the ATPase in vivo, and can be destroyed by trypsin treatment.

3. ATP and ADP are found tightly bound to the coupling ATPase of *P. denitrificans*, both in its membrane-bound and isolated state. The ATP/ADP ratio on the enzyme is greater than one.

4. Under de-energised conditions, the bound nucleotides are not available to the suspending medium. When the membrane is energised however, the bound nucleotides can exchange with added nucleotides and incorporate $^{32}P_i$. $^{32}P_i$ is incorporated into the β and γ positions of the bound nucleotides, but β-labelling probably does not occur on the coupling ATPase.

5. Uncouplers inhibit the exchange of the free nucleotides or $^{32}P_i$ into the bound nucleotides, while venturicidin (an energy transfer inhibitor) and aurovertin stimulate the exchange.

6. The response of the bound nucleotides to energisation is consistent with their being involved directly in the mechanism of oxidative phosphorylation.

INTRODUCTION

Paracoccus denitrificans (formerly known as *Micrococcus denitrificans*) [1] is a free-living bacterium which is incapable of fermentation and thus depends entirely on respiration as a source of energy. It can use either free oxygen or nitrate as a terminal

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Abbreviations: S_{13}, 5-chloro-3-*t*-butyl-2′-chloro-4′-nitrosalicylanilide; P/O ratio, mol P_i esterified per mol O consumed.

electron acceptor. Like those from *Azotobacter vinelandii* [2], coupled membrane fragments can be prepared from *P. denitrificans* [3, 4], oxidation of NADH being stimulated 2–3 times by the addition of ADP and P_i. The maximum observed rate of phosphorylation by these membranes (about 1 μmol ATP synthesised/min per mg protein) is considerably higher than the rate of ATP hydrolysis (5–10 nmol ATP hydrolysed/min per mg membrane protein).

Such properties make the membranes of *P. denitrificans* especially suitable for studying the mechanism of coupling in oxidative phosphorylation. Compared to those of mitochondria and chloroplasts, however, little is known about the coupling system of *P. denitrificans*. It is shown here that the coupling ATPase of this organism and its interaction with the membrane is similar to those in other coupled membranes.

In particular, this ATPase bears especially tight binding sites for ATP and ADP. Nucleotides are found bound to these sites at levels on the membrane of 1.1 nmol ATP and 0.9 nmol ADP per mg membrane protein. These nucleotides are not normally available to the ambient solution but, on energisation of the membrane, they exchange with nucleotides in solution and can incorporate $^{32}P_i$ from the solution, even in the absence of added nucleotides.

All coupling ATPases so far investigated, from oxidative and photosynthetic systems, bear tightly bound ATP and ADP, but until now only in photosynthetic systems have these nucleotides been shown to respond to energisation [5]. The purpose of this and the accompanying paper [6] is to demonstrate that tightly bound nucleotides of the coupling ATPase are involved in phosphorylation also in oxidative systems. Two systems were used. In the first, the aerobic bacterium *P. denitrificans*, only electron transport will serve to energise the membrane. Just as in untreated chloroplasts, the ATPase activity of the membrane is insufficient to cause energisation. In the second, beef heart mitochondria, the membrane can be energised either by ATP or by oxidisable substrate.

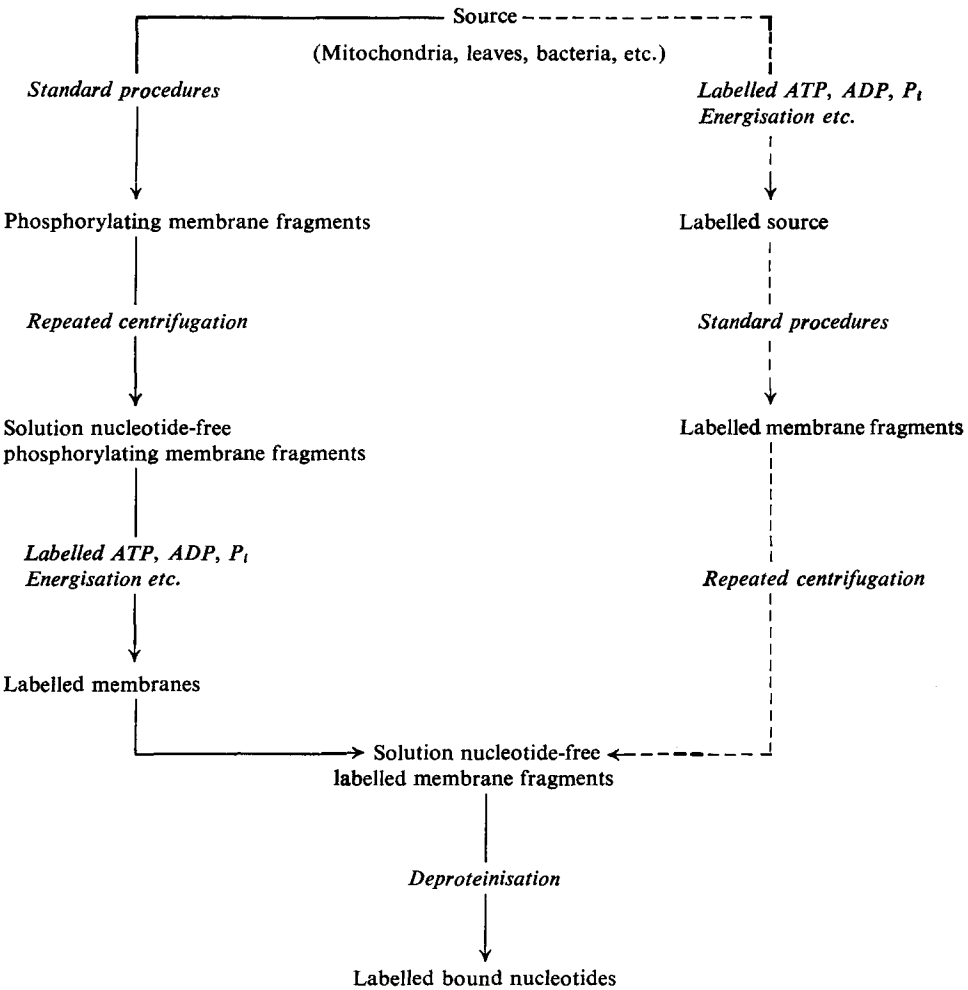
In both systems used, the bound nucleotides of the coupling ATPase respond in an analogous way to the chloroplast system [5] i.e. they become exchangeable on energisation. This is consistent with a general model for phosphorylation in which energy is not required for ATP sythesis, but to release bound ATP from the membrane [7, 8]. The bound nucleotides can also serve as an intrinsic probe of energisation of the coupling ATPase.

MATERIALS AND METHODS

Coupled, 'inside-out' vesicles were prepared from *P. denitrificans* as described by Burnell et al. [9] except that 10 mM ATP was added to the 100 mM Tris acetate in which the lysozyme-treated cells were suspended prior to lysis. ATPase activity of these vesicles was measured as described by Imai et al. [10].

The coupling ATPase was removed from the membrane by washing at low salt concentrations, by a modification of the procedure of Strotmann et al. for chloroplasts [11]. P/O ratios were measured using $^{32}P_i$, monitoring oxygen uptake polarographically [12].

Nucleotide assays were performed on deproteinised extracts of membranes or purified enzymes as described previously [5, 13]. It was essential to treat radioactively labelled ATP with phospho*enol*-pyruvate and pyruvate kinase before use to avoid



Scheme I

interference by small amounts of (carrier-free) ADP [13]. Insoluble protein was measured by the biuret procedure of Cleland and Slater [14] and soluble protein by the method of Lowry et al. [15].

Experiments to study the behaviour of the bound nucleotides under energised conditions were performed as in Scheme I (cf. also ref. 5). The preferred design is that shown on the left of Scheme I (solid arrows). Phosphorylating membrane fragments, prepared by standard techniques, are washed by repeated centrifugation to remove their free nucleotide pool. The washed particles (solution nucleotide-free) have a known complement of (bound) nucleotides and can be energised in the presence of a given labelled nucleotide or P_i alone. After energisation (or other treatment) the free nucleotides are again removed, allowing the bound nucleotides to be investigated for distribution of label, etc.

In some cases, especially in bacterial systems, it is not possible to prepare well

coupled membrane fragments and/or the membranes may become uncoupled during washing. In this case, it is still possible to investigate some activities of the bound nucleotides using the procedure on the right of Scheme I (dashed arrows), as used for example in *Streptococcus faecalis* by Abrams et al. [16]. The main problem with this approach is that, at the time of labelling, the nucleotide and/or P_i pools are not well defined.

Venturicidin was a kind gift of Dr. D. Griffiths, University of Warwick, and aurovertin of Dr. R. M. Bertina, University of Amsterdam. Other compounds were obtained as previously [5].

RESULTS

Sensitivity of P. denitrificans vesicles to washing procedures

The coupling of vesicles from *P. denitrificans* is affected by the medium with which they are washed. The P/O ratios are decreased by washing vesicles in a medium lacking Mg^{2+} and/or ATP (Table I). The ATPase activity (not shown) and the bound nucleotide content (below) of these membranes is not altered by this procedure. We thus conclude that no loss of coupling ATPase occurs during these washes, but Mg^{2+} and ATP are required in addition to maintain a fully coupled state of the membrane.

A more drastic fall in P/O ratio is seen if the membrane is washed at very low salt concentrations (2 mM Tris as the only cation) with a small amount of EDTA present (Table I). Such conditions favour the dissociation of the coupling ATPase from the membranes of other bacteria [17].

TABLE I

SENSITIVITY OF *P. DENITRIFICANS* MEMBRANES TO WASHING

P. denitrificans membranes were suspended at about 5 mg protein/ml in 10 mM Tris acetate, 1 mM magnesium acetate. These were 'unwashed particles'. Washed particles were prepared by diluting these particles in the buffers indicated to about 100 μg/ml and centrifuging down at 40000 $\times g$ for 20 min at 0 °C. The particles were then resuspended in 10 mM Tris acetate, 1 mM magnesium acetate. All buffers were at pH 7.4. P/O ratios were measured using NADH as substrate in 50 mM Tris acetate, 5 mM magnesium acetate after 5 min preincubation in this medium, except in Expt. B, (3)*, when there was no preincubation. In all other cases, preincubation had little (< 5 %) effect on the P/O ratios.

Expt. A		Expt. B	
Wash medium	P/O ratio	Wash medium	P/O ratio
Unwashed	1.4	(1) Tris acetate 10 mM Mg acetate 1 mM	0.49
Tris acetate 10 mM Mg acetate 1 mM ATP 1 mM	1.4	(2) EDTA (sodium) 20 mM	0.28
Na_4PP_i 20 mM	0.89	(3) EDTA (sodium) 20 mM ATP 1 mM	0.47 (0.32*)
Tris acetate 10 mM Mg acetate 1 mM	0.71		
EDTA (Tris) 0.5 mM	0.09		

Higher concentrations of EDTA (20 mM) were much less effective at decreasing the P/O ratio, and the depression could be at least partially restored by incubation of the particles with Mg^{2+}. Thus, loss of coupling was not simply due to removal of Mg^{2+} from the membrane, but in addition required low salt conditions.

2.5 M LiCl also abolished phosphorylation ([10], see below). None of the washing procedures used above inhibited oxygen uptake.

Isolation of the coupling ATPase of P. denitrificans

P. denitrificans membranes contain two distinct ATPase activities [10, 12]. One is stimulated by bicarbonate ions and inhibited by venturicidin, ($K_{\frac{1}{2}}$ = 50 ng/mg protein) and this appears to be the coupling ATPase since both it and phosphorylation are inhibited by venturicidin [18] and 7-chloro-4-nitrobenzo-2-oxa-1,3 diazole [19]. (Oligomycin does not inhibit either process in these membranes). The role of the second ATPase, which has a different pH profile and metal ion requirement [12], is uncertain.

Washing *P. denitrificans* membranes at low salt concentrations specifically removes both the venturicidin-sensitive ATPase (Table II) and the coupling (Table I). This confirms that it is this ATPase that is involved in ATP synthesis. Washing the membranes with 2.5 M LiCl leads to the loss of both ATPases, in agreement with the observations of Imai et al. [10]. The supernatant from the LiCl wash contains no active ATPase, but that from the low salt wash does. If either type of washed particle is incubated with excess supernatant from the low salt wash, in the presence of Mg^{2+}, the venturicidin-sensitive ATPase is restored (Table II). Some non-specific binding probably also occurs, since the venturicidin-insensitive ATPase also rises. (Phos-

TABLE II

REMOVAL OF THE COUPLING ATPase FROM *P. DENITRIFICANS* MEMBRANES, AND RECONSTITUTION

P. denitrificans membranes were washed by centrifugation through 0.5 mM EDTA (Tris), 10 % glycerol, pH 7.4, or through 2.5 M LiCl, as described in Table I. Aliquots of the two types of washed membranes were incubated with excess supernatant from the EDTA-glycerol wash (*n* mg of membranes with the supernatant from 2*n* mg), with the addition of 4 mM $MgCl_2$, 1 mM ATP, pH 7.4, at 30 °C for 5 min. The membranes were spun down again, and resuspended in 10 mM Tris acetate, 1 mM magnesium acetate, pH 7.4. Venturicidin, where present, was added at 5 μg/mg protein. (95 % of maximal inhibition of the membrane ATPase is achieved at 500 ng/mg protein, Ferguson, S. J. and John, P., unpublished results.) 'Control' particles were incubated with the EDTA-glycerol medium, but before centrifugation, 4 mM $MgCl_2$, 1 mM ATP, pH 7.4 was added to the solution. (Similar results were obtained in the absence of glycerol, not shown.)

Treatment	ATPase activity (nmol · min^{-1} · mg $protein^{-1}$)		Venturicidin-sensitive ATPase (nmol · min^{-1} · mg $protein^{-1}$)
	−Venturicidin	+Venturicidin	
None	9.0	4.6	4.4
'Control'	10.6	7.1	3.5
Low salt wash (1)	6.4	5.6	0.8
LiCl wash (2)	2.8	3.8	(−1.0)
(1), Reconstituted	14.7	8.3	6.4
(2), Reconstituted	13.1	8.8	4.7

phorylation is not restored with the ATPase activity. Possibly some other damage to the membranes occurs during the stripping procedure).

We have thus demonstrated a mild procedure for removing the coupling ATPase from *P. denitrificans* membranes. The specific activity of the isolated ATPase immediately after isolation is about 60 nmol ATP/min per mg protein (six times that of the membrane-bound ATPase), but it decreases during purification and no effective stabilising treatment has yet been found.

Preliminary characterisation of the ATPase of P. denitrificans

Although the coupling ATPase isolated by this procedure is impure, as shown by polyacrylamide gel electrophoresis, it has been possible to demonstrate several properties in common with other isolated coupling ATPases (cf. refs. 17, 20).

(1) Gel filtration shows the molecular weight of the ATPase to be about 300 000.

(2) Electrophoresis shows that the ATPase is highly negatively charged.

(3) The major subunits in the impure ATPase preparation have a molecular weight of around 50 000, as shown by electrophoresis in dodecyl sulphate [12].

(4) The ATPase is stimulated by trypsin (Fig. 1). This is common to other coupling ATPases [2, 21, 22] and is generally taken to indicate the presence of a trypsin-sensitive ATPase inhibitor protein bound to the ATPase [22]. As in *E. coli*, the stimulation is only about two-fold. Higher concentrations of trypsin, or more prolonged treatment, inhibit the ATPase. The coupling ATPase of *P. denitrificans* has low activity (50–100 nmol/min per mg protein) compared to mitochondrial ATPase (100 μmol/min per mg protein). This characteristic is shared with other aerobic bacteria and chloroplasts [17, 20]. This may be related to the fact that in these organisms ATP is not used to drive membrane-linked processes in vivo, unlike in mitochondria and facultative or obligate anaerobes [23].

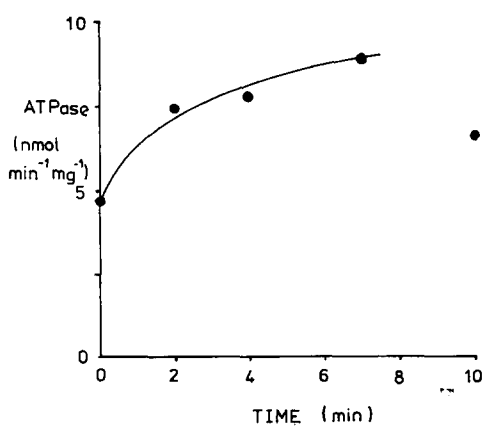


Fig. 1. Stimulation of *P. denitrificans* ATPase by trypsin. *P. denitrificans* membranes (2.5 mg protein) were incubated at 30 °C in a solution containing 12 μmol ATP, 15 μmol EDTA, 100 μmol Tris acetate buffer (pH 7.4), 0.6 mg trypsin in a total volume of 3 ml. At the times indicated, 0.4 ml aliquots were treated with 0.4 mg trypsin inhibitor (soybean) and the venturicidin-sensitive ATPase measured.

TABLE III

BOUND NUCLEOTIDES OF *P. DENITRIFICANS* MEMBRANES

Membranes were isolated from *P. denitrificans* and washed by centrifugation (40000 × *g*, 20 min.) through (a), 10 mM Tris acetate, 1 mM magnesium acetate, pH 7.4; 4 times; (b), 20 mM sodium pyrophosphate, pH 7.4; 4 times; (c), buffer (b) 3 times, followed by 0.5 mM EDTA (Tris), pH 7.4; (d), buffer (a) 3 times, followed by the same buffer containing 10 mM glucose and 4 U hexokinase per ml. (1 U = 1 μmol · min^{-1}); (e), buffer (b) 4 times; (f), buffer, (a) 4 times, except that the membranes were incubated at 100 °C for 15 min prior to washing; (g), supernatant from (c), final wash, concentrated in an Amicon UM-50 ultra-filter cone. The washed protein was then extracted with perchloric acid as described, except that in sample (e) 2 % dodecyl sulphate was added to dissolve the membranes prior to perchloric acid treatment, and 1 mg/ml albumin to the extract (to adsorb the detergent) prior to assay. Results are given in nmol per mg protein.

Sample	ATP	ADP	AMP
(a) Tris-washed membranes	1.1	0.9	0.5
(b) PP_i-washed membranes	1.4	1.1	0.5
(c) 'ATPase-stripped' membranes	0.9	—	—
(d) Glucose/hexokinase membranes	1.4	1.2	—
(e) Dodecyl sulphate extracted membranes	1.0	1.3	—
(f) Boiled membranes	<0.1	<0.1	—
(g) Coupling ATPase fraction	3.0	—	—

Bound nucleotides of the coupling ATPase of P. denitrificans

Table III shows that washed membranes of *P. denitrificans* contain tightly bound nucleotides. The nucleotides are completely removed from the membrane by the perchloric acid extraction used; no further nucleotides are released by detergent treatment (cf. ref. 5). Denaturation of the membranes, by heating at 100 °C for 15 min, also removes the nucleotides from the membrane, confirming that they are bound non-covalently and not by some acid-labile bond.

The nucleotides are not removed by washing the membranes in pyrophosphate solutions (which removes non-specifically bound nucleotides very effectively) nor by treatment with glucose and hexokinase. They are, however, partially removed by washing at low salt concentrations (which partially removes the coupling ATPase

TABLE IV

AVAILABILITY OF BOUND NUCLEOTIDES TO LUCIFERASE

An extract of firefly tails containing luciferin-luciferase was incubated in 3 ml water containing 100 μmol sodium arsenate, 10 μmol Tris base, 44 μmol $MgSO_4$ brought to pH 7.4 with H_2SO_4. The light emitted was measured in a scintillation counter [24]. To this solution was added about 0.5 mg washed *P. denitrificans* membranes, and the light emitted again measured. To the same sample, 40 pmol ATP was added as an internal standard, and the light again measured.

Addition	Counts in 6 s	Free ATP (pmol)	Bound ATP* (pmol)
None	120	1	0
+0.5 mg *P. denitrificans* membranes	1300	10	700
+20 μl 2 μM ATP	6250	50	700

* Estimated value.

TABLE V

EXCHANGE OF BOUND NUCLEOTIDES ON *P. DENITRIFICANS* MEMBRANES

Membrane vesicles from *P. denitrificans* were washed twice by centrifugation through (a) 10 mM Tris acetate, 1 mM Mg acetate, 1 mM ATP, pH 7.4; (b) and (c) 10 mM Tris acetate, 1 mM Mg acetate, pH 7.4. They were then incubated in 50 mM Tris acetate, 5 mM magnesium acetate, pH 7.4, for 5 min at 30 °C with shaking in the presence of (a) 1 mM [^{3}H]ATP (4000 cpm/nmol); (b) 0.5 mM [^{3}H]ADP (2000 cpm/nmol); (c) 2 mM $^{32}P_i$ (7500 cpm/nmol); with or without 2 mM NADH as indicated. The protein concentration was 1–2 mg/ml. The particles were then washed by centrifugation through 10 mM Tris acetate, 1 mM magnesium acetate until no further counts were present in the supernatant, and finally resuspended at 4–5 mg protein/ml in 10 mM Tris acetate 1 mM EDTA for nucleotide analysis as described. For the 'stripped' particles, 0.5 mM EDTA neutralised with Tris to pH 7.4 replaced the Mg^{2+}-containing buffer in the last wash.

Incubation with	Label incorporated (nmol/mg protein)	Label/total nucleotide (mol/mol AdN bound)
(a) [^{3}H] ATP	0.16	0.10
+NADH	0.83	0.55
+NADH, 'stripped'	0.50	0.50
(b) [^{3}H] ADP	0.06	—
+NADH	0.25	—
(c) $^{32}P_i$	0.03	0.02
+NADH	0.20	0.14
+NADH, 'stripped'	0.13	0.13

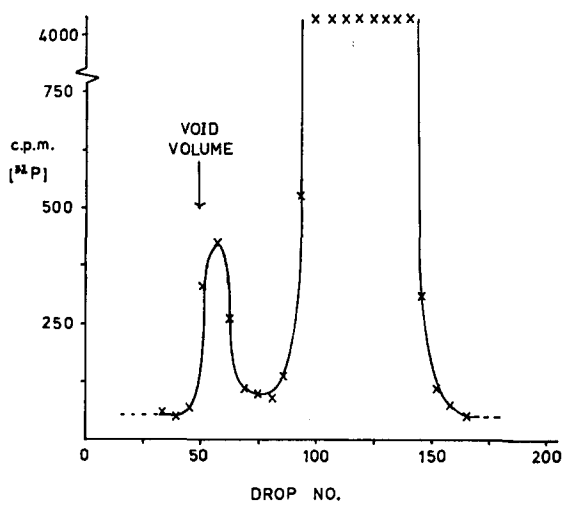


Fig. 2. Protein-bound radioactivity in coupling ATPase fraction of labelled *P. denitrificans* membranes. A labelled 'coupling ATPase fraction' was prepared as in Table V, Expt. c, except that the membranes were washed only twice before stripping. The supernatant from the low-salt wash centrifuged for 90 min at 100000 × *g* to remove membrane fragments, and concentrated about ten fold in an Amicon UM-50 ultrafilter cone. 0.5 ml of the concentrate was applied to a Sephadex G25 column (6 × 1 cm) previously equilibrated with a buffer containing 250 mM sucrose, 10 mM Tris acetate, 2 mM EDTA, 1 mM ATP at pH 7.4, and eluted with this buffer. Fractions were counted for ^{32}P-label.

from the membrane, see Table II) and are recovered in a protein-bound state in the coupling factor fraction. It seems likely, therefore, that *P. denitrificans*, like other coupled systems, has very tight binding sites for nucleotides on its coupling ATPase. It should be noted that there is no translocator for adenine nucleotides in these particles, and so the translocase cannot represent a tight binding site for nucleotides here.

Table IV shows that, as in mitochondria [6], the bound nucleotides are not available to enzymes in the ambient solution. No additional ATP is released if substrate (NADH) is added to the solution, unless ADP is also present (not shown).

Exchangeability of bound nucleotides in P. denitrificans membranes

The responses of the bound nucleotides to energisation were investigated as in Scheme I, (solid arrows). Investigation was hampered in this system because, as shown in Table I, washing *P. denitrificans* membranes to remove ATP results in partial loss of coupling. To alleviate this problem, membranes to be incubated with labelled ATP were washed in buffers containing low concentrations of ATP (100 μM) and so retained their coupling. Particles to be incubated with labelled ADP and/or P_i were washed without ATP and so were less well coupled.

Table V shows that energisation of the membranes by electron transfer leads to exchange of the bound with free nucleotides, and incorporation of $^{32}P_i$ into bound nucleotide, as in chloroplasts [5]. $^{32}P_i$ incorporation into the bound nucleotides does not require addition of ADP, and is unaltered if ADP is added during energisation (not shown). Removal of the coupling ATPase, by washing the membranes at low salt concentrations, causes loss of total and labelled nucleotides such that the ratio of labelled to total nucleotide on the membranes remains constant. Thus the labelled and total nucleotides behave identically during the stripping procedure, as if they comprised a uniform pool of nucleotide.

The nucleotides released in the low salt wash are protein-bound, for they elute from a Sephadex G-25 column at the void volume (Fig. 2). Similar results are obtained whether $^{32}P_i$ (Fig. 2) or [^{3}H]ATP (not shown) is used for labelling. This is consistent with the nucleotides being labelled on the coupling ATPase and remaining tightly bound to it during its removal from the membrane.

Complete exchange of all the bound nucleotides does not occur in *P. denitrificans* (Table V) and mitochondrial membrane vesicles ([6], Table V), as it does in chloroplasts [5]. It seems likely that this is because these membrane preparations are less well coupled than preparations of chloroplast membranes. It is assumed that if these membranes were perfectly coupled, all their nucleotides would be exchangeable, as occurs in chloroplasts. The reason for the high 'basal' (non-energised) exchange in submitochondrial particles relative to the other systems is uncertain.

Distribution of label among the bound nucleotides

Table VI shows the distribution of radioactivity among the bound nucleotides, after labelling with [^{3}H]ATP. ATP, ADP and AMP are labelled to a similar extent (AMP is smallest in amount and probably a contaminant due to the myokinase activity of these membranes [12]). More than 98 % of the counts released on denaturation of the membrane are absorbed by charcoal and more than 98 % of the counts applied to the chromatogram are recovered as ATP, ADP and AMP, confirming that

TABLE VI

DISTRIBUTION OF LABEL IN BOUND NUCLEOTIDES AFTER INCUBATION WITH [^{3}H]ATP

Labelled particles were prepared as in Table V (a), except that [^{3}H]ATP at 16000 cpm/nmol was used. Total bound nucleotide and distribution of label were measured as described [5].

	Percent of total measured		
	ATP	ADP	AMP (bound)
Radioactivity as:	47	32	21
Nucleotides as:	47	35	18

negligible transfer of label occurs from [^{3}H]ATP into molecules other than adenylate esters in these incubations.

Table VII shows that, as in chloroplasts [5], $^{32}P_i$ labels both the β and γ positions of bound ATP and ADP equally. During net phosphorylation, however, only γ-labelled ATP is released to the solution. It is probable that β-labelling of the bound nucleotides does not occur on the coupling ATPase, as discussed later.

Effect of phosphorylation inhibitors on the exchange of bound nucleotides

Table VIII shows that inhibitors of phosphorylation affect the energised exchange of the bound nucleotides in *P. denitrificans*, as in chloroplasts [5] and mitochondria [6]. Uncouplers, such as gramicidin+ammonia, and S_{13} inhibit exchange of the bound ATP with added ADP, and of $^{32}P_i$ into the bound nucleotides. Unlike chloroplasts, inhibition by uncouplers was not complete.

The 'energy-transfer' inhibitor venturicidin does not inhibit (indeed it stimulates) the exchanges, at concentrations sufficient to inhibit phosphorylation completely. Thus energisation of the ATPase by electron transfer is not completely inhibited by venturicidin. This is also the case in mitochondria [6]. The venturicidin-induced

TABLE VII

DISTRIBUTION OF ^{32}P LABEL IN BOUND AND FREE NUCLEOTIDES DURING PHOSPHORYLATION WITH $^{32}P_i$

^{32}P-labelled particles were prepared as in Table V (c). Phosphorylation was carried out in the same medium, except that 1 mM ADP was present, the protein concentration was 50 μg/ml and the specific activity of the $^{32}P_i$ was 700 cpm/nmol. Duplicate nucleotide extracts were prepared from each sample, and one treated with hexokinase (10 U), (1 U = μmol · min^{-1}), glucose(20 mM) and $MgCl_2$ (5 mM) prior to charcoal extraction and chromatography. Results are expressed as percent counts in (ATP+ADP) before hexokinase treatment.

	Percent total radioactivity in:			
	before hexokinase		after hexokinase	
	ATP	ADP	ATP	ADP
Bound nucleotides	65	35	35	48
Free nucleotides	94	6	0	5

TABLE VIII

INHIBITOR SENSITIVITIES OF THE REACTIONS OF THE BOUND NUCLEOTIDES IN *P. DENITRIFICANS*

P. denitrificans membranes were labelled by $^{32}P_i$ or [^{3}H]ATP as described in Table V except that various inhibitors of phosphorylation were added as indicated. Incorporation was measured as described and expressed as percent value obtained in the absence of inhibitors.

Addition	Incorporation of label (%)	
	[^{3}H]ATP	$^{32}P_i$
None	100	100
gramicidin (4 μg/mg)+NH_4Cl (100 mM)	59	58
S_{13} (30 nmol/mg)	—	61
Venturicidin (1 μg/mg)	142	139
Aurovertin (1 μg/mg)	131	118
EDTA (4 mM)	44	18

stimulation of the exchanges is probably due to a 'recoupling' effect of the inhibitor on partially coupled particles, as is seen for oligomycin in mitochondria [25].

Aurovertin inhibits phosphorylation in *P. denitrificans* after a preliminary incubation of the membranes with this antibiotic (Ferguson S. J., Harris, D. A., Lloyd, W. J. and John, P., unpublished observations). As in mitochondria, it stimulates rather than inhibits the exchange reactions of the bound nucleotides at concentrations which inhibit phosphorylation by more than 70 %. The explanation for this finding is discussed in the accompanying paper [6].

As is the case with chloroplasts [5], EDTA is the only inhibitor tested in *P. denitrificans* which inhibits the nucleotide and $^{32}P_i$ exchanges to different extents (Table VIII). The exchange against $^{32}P_i$ is the more strongly inhibited, presumably because it requires free Mg^{2+}. In *P. denitrificans*, the nucleotide exchange is also inhibited to some extent by EDTA, but this is probably a secondary effect due to the uncoupling action of EDTA (Table I).

DISCUSSION

Bound nucleotides of coupling ATPases

It is demonstrated here that coupled *P. denitrificans* membrane vesicles bear a coupling ATPase similar to that of other coupling membranes. This coupling ATPase binds ATP and ADP especially tightly, and continues to bind them when removed from the membrane. It seems likely [6] that all coupling ATPases bear about 2 mol ATP/mol and 1 mol ADP/mol, and that the coupling ATPase is the only major site for such tight binding of nucleotides in coupled membranes.

Thus we can estimate the amount of coupling ATPase in *P. denitrificans* from the amounts of ATP or ADP bound to the membranes. This value is about 0.6–0.9 nmol/mg membrane protein (depending on whether the amount of ATP or ADP is taken), about 1.5–2 times the value for submitochondrial particles.

Independently of their role in phosphorylation, nucleotides bound to the coupling ATPase provide us with a specific, intrinsic probe of this enzyme. These

results demonstrate that, on energisation, a conformational change occurs in the coupling ATPase in oxidative phosphorylating systems. This has been previously demonstrated only in photosynthetic systems [5, 26, 27]. This change occurs in the coupling ATPase itself, and is not simply an exposure of part of the coupling ATPase due to, for example, a shift in the inhibitor protein (as was possible from the earlier data [26, 27]). Uncouplers prevent this change, while EDTA does not affect the conformational change but prevents one of the subsequent steps of phosphorylation [5].

The major problem with techniques such as those reported here and those of earlier workers [26, 27] is their lack of resolution in time. In particular, a compound which inhibits an ATPase molecule 99 % of the time (i.e. a readily reversible inhibitor) will not inhibit the exchange reactions measured here nearly as well as one which inhibits 99 % of the ATPases permanently (a slowly reversible inhibitor), since either a few, or very many turnovers of an ATPase will lead to the same total exchange. This must be borne in mind when interpreting the results obtained with venturicidin and aurovertin, both of which inhibit phosphorylation at the concentrations used here, but not the exchange reactions. Thus, either compound might act to prevent the conformational change of the ATPase involved in phosphorylation, but with rapid reversibility, or might allow such a conformational change but prevent a later step in phosphorylation. This is discussed further in the accompanying paper [6].

β-Labelling of the bound nucleotides

Incubation of energised particles with $^{32}P_i$, in the presence or absence of added nucleotide, leads to labelling of the bound nucleotides in the β and γ positions. This occurs in chloroplasts [5], mitochondria [6], *Rhodospirillum rubrum* [28] and *P. denitrificans* (above) and so appears to be general in phosphorylating systems. We previously suggested that this labelling was on a side path to phosphorylation [5], and this view has been confirmed by Boyer and co-workers, who show that β-labelling is too slow to be involved in phosphorylation, in chloroplasts and mitochondria at least. [29].

β-Labelling here is probably due to myokinase and ADP $\rightleftharpoons$ P_i exchange enzymes acting in concert with the coupling ATPase, and randomising the label incorporated. These enzymes are present in sufficient amounts in washed *P. denitrificans* membranes [12]. It seems, however, that they must be very closely associated with the coupling ATPase since added ADP does not diminish β-labelling of the bound nucleotides (above) and the mechanism of β-labelling is not yet completely explained.

Bound nucleotides and oxidative phosphorylation

The main purpose of these experiments was to investigate a possible role of bound nucleotides of the coupling ATPase in oxidative phosphorylation. It is shown that tightly bound nucleotides are found in oxidative as well as photo-phosphorylating systems. In all cases, ATP levels exceed ADP levels, consistent with the view that the ATPase with ATP bound to it represents a low energy rather than a high energy state. No evidence is found, in submitochondrial particles [6] or *P. denitrificans* for a second (hydrophobic) nucleotide-binding compartment whence the nucleotides are released by acid only in the presence of a detergent. Some workers have found such a

compartment in photosynthetic systems [28, 30]. It is concluded that, if such a compartment does exist (and is not merely due to the difference in extraction procedures used), this compartment is not important in a mechanism of phosphorylation common to oxidative and photosynthetic systems.

It is also demonstrated above for *P. denitrificans* and in the accompanying paper [6] for submitochondrial particles, that the bound nucleotides of the coupling ATPase in oxidative phosphorylative systems respond to energisation of the membrane. On energisation, they become less tightly bound to the ATPase. This is consistent with a general mechanism of phosphorylation [5–8] in which energy is not required for ADP-P_i bond formation itself but for ATP release from the ATPase. A possible role for the various nucleotide sites of the ATPase is suggested in the following paper.

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