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Adenosine 5'-Triphosphate-Linked Transhydrogenase in Cytoplasmic Membranes of Colicin-Treated and Untreated *Escherichia coli*

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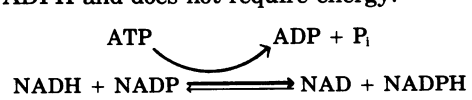
The adenosine 5'-triphosphate (ATP)-linked transhydrogenase reaction, present in the particulate fractions of *Escherichia coli*, was previously shown to be inhibited in these fractions when the bacteria were treated with colicins K or E1. The purpose of this study was to characterize the ATP-linked transhydrogenase reaction and the colicin-caused inhibition of the reaction in purified cytoplasmic membranes. Particulate fractions from bacteria treated or untreated with colicins were separated on sucrose gradients into cell wall membrane and cytoplasmic membrane fractions. The ATP-linked transhydrogenase reaction was found to be exclusively associated with the cytoplasmic membrane fractions. The reaction was inhibited by carbonylcyanide *m*-chlorophenylhydrazone, dinitrophenol, *N,N'*-dicyclohexylcarbodiimide, and trypsin. Although the cytoplasmic membrane fractions were purified from the majority of the cell wall membrane and its bound colicins, they showed the inhibitory effects of colicins K and E1 on the ATP-linked transhydrogenase reaction. The inhibition of ATP-linked transhydrogenase reaction induced by the colicin could not be reversed by subjecting the isolated membranes to a variety of physical and chemical treatments. Cytoplasmic membranes depleted of energy-transducing adenosine triphosphatase (ATPase) complex (coupling factor) lost the ATP-linked transhydrogenase activity. The ATPase complexes isolated from membranes of bacteria treated or untreated with colicins E1 or K reconstituted high levels of ATP-linked transhydrogenase activity to depleted membranes of untreated bacteria. The same ATPase complexes reconstituted low levels of activity to depleted membranes of the treated bacteria.

Although considerable progress has been made in elucidating the mode of action of colicins, our understanding of the events underlying the observed effects of these bactericidal proteins is still incomplete (for a recent review see reference 11). This is particularly true in the case of colicins K and E1. Both colicins inhibit a variety of functions in intact bacteria (7, 13, 16, 21). It is generally thought that these colicins affect cell functions through their effects on the cell membrane (6, 14, 21), yet only a few studies with isolated membranes have been made. In one study (1), colicin E1 or K was added to vesicles of *Escherichia coli*, which were prepared according to the method of Kaback, and this resulted in inhibition of amino acid transport (1). In a more recent study (23), particulate fractions prepared from bacteria treated with colicin E1 or K were found to be impaired in their ability to catalyze the adenosine 5'-triphosphate (ATP)-linked transhydrogenase reaction. This reaction (represented below by the heavy arrow) involves the reduction of nicotinamide adenine dinucleotide phosphate (NADP) by reduced nicotinamide adenine dinucleotide (NADH) coupled to the hydrolysis of ATP. The reaction in the opposite direction (represented below by the light arrow) is termed the NADPH-NAD transhydrogenase reaction. It involves reduction of NAD by NADPH and does not require energy.



The particulate fractions, which were previously used to study the ATP-linked transhydrogenase reaction (23), consist of bacterial cell wall membrane, the locale of colicin receptors (25), and cytoplasmic membrane, the site of energy-transducing processes.

The purpose of the present investigation was

to characterize the ATP-linked transhydrogenase reaction in purified cytoplasmic membranes and to determine whether the effect of colicin E1 or K on this reaction is maintained in these membranes. Several approaches were utilized to further characterize the colicin-caused inhibition of the ATP-linked transhydrogenase reaction.

MATERIALS AND METHODS

Unless otherwise stated, materials and methods were as described previously (23).

Chemicals. The following chemicals were purchased from Sigma Chemical Co: *N,N'*-dicyclohexylcarbodiimide (DCCD), carbonylcyanide *m*-chlorophenylhydrazone (CCCP), and dinitrophenol (DNP).

Media and growth conditions. For membrane preparations, bacteria were routinely grown to logarithmic phase in LB media (15). Bacteria grown under anaerobic conditions were cultivated in LB media supplemented with the following (in grams per 100 ml): glucose, 0.2; Na_2CO_3, 0.4; cysteine, 0.05. The pH of the supplemented media was adjusted to neutrality with sterilized 6 N HCl. Flasks containing the anaerobic media were flushed extensively with nitrogen gas before and after inoculation with bacteria. The inoculated flasks were sealed under nitrogen and incubated undisturbed at 37°C. After incubation for 18 h, the cultures reached a density of 4×10^8 to 6×10^8 cells/ml. Anaerobic cultures, which were shifted to aerobic conditions, were plugged with cotton and shaken for 2 h at 37°C. This period of growth allowed the cultures to double their cell number.

Colicin preparation. Purified colicins were used throughout this study. Induction, extraction, and purification of colicins were carried out according to the methods described by Herschman and Helinski (10) for colicins E2 and E3 and according to Kunugita and Matsuhashi (12) for colicin K (this method was also used for the preparation and purification of colicin E1). The diethylaminoethyl-Sephadex purification step of the latter method was omitted. Unless otherwise stated, colicin treatment of aerobic cultures was carried out for 5 min at 37°C. Anaerobic cultures were treated for 10 min at 37°C under nitrogen. Colicins were used at multiplicities of 5 killing units or less. Survival assays following colicin treatments were carried out in the presence of trypsin (23).

Preparation of cytoplasmic and cell wall membranes. Particulate fractions prepared as described previously (23) were suspended in 0.01 M *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid buffer (HEPES) of pH 7.4 with 0.005 M ethylenediaminetetraacetate (EDTA) and 20% sucrose. Samples containing 20 to 40 mg of protein were layered on stepwise-formed sucrose gradients and centrifuged in an SW27 rotor (Beckman) at 22,000 rpm for 18 h at 2 to 4°C. The gradients were formed by layering the following sucrose solutions (wt/vol): 70% (3 ml), 60% (3 ml), 58% (3 ml), 55% (3 ml), 53% (3 ml), 50% (9 ml), 48% (3 ml), 45% (3 ml), 40% (3 ml), and 35% (1 ml). The sucrose solutions were prepared in 0.01 M HEPES buffer (pH 7.4) with 0.005 M EDTA. Inclusion of EDTA in the suspensions and in the gradient solutions was found necessary to achieve a better separation of the cell wall membrane from the cytoplasmic membrane. Fractions (80 drops) were collected from the bottom of the gradient tubes with the aid of a Buchler peristaltic pump. The absorbancy of the fractions was measured at 280 nm. The peak tubes were pooled and frozen in an acetone–dry-ice bath in 0.5- to 1-ml portions and stored frozen at −20°C. Samples were thawed just before assays.

Biochemical assays. The energy-dependent transhydrogenase reaction was assayed as described in reference 23. Sucrose was omitted from the assay mixture of this reaction when the assay was performed on sucrose gradient fractions. Formation of NADPH was measured as the increase in absorbance at 340 nm in a Zeiss spectrophotometer. Other enzyme measurements were carried out as previously described (23). Units of enzyme activity were calculated as unit changes in optical density per minute and were measured from the initial rates at 340 nm for NADH oxidase and NADPH-NAD transhydrogenase and at 550 nm for succinic dehydrogenase. The adenosine triphosphatase (ATPase) units were calculated as micromoles of inorganic phosphorus released per minute.

Colicin receptor assay. The colicin receptor assay was performed as described previously (24), using the colicin neutralization spot test. Volumes of samples to be tested for receptor activity were incubated with equal volumes of serial twofold dilutions of colicin E3. The samples of cell fractions contained protein in the range of 0.1 to 1 mg/ml. Units of receptor activity are expressed as arbitrary neutralization units per milligram of sample protein (24).

Effect of uncouplers and inhibitors on energy-dependent transhydrogenase. Solutions of CCCP, DNP, or DCCD were prepared in methanol and added to assay mixtures at the beginning of the 20-min incubation period (see reference 23). Control assay mixtures received an equivalent volume of methanol.

Trypsin treatment of membranes. Trypsin was added to membrane fractions at a protein concentration equal to the protein concentration of the membranes and incubated at room temperature for 10 min. Trypsin was then inactivated by the addition of soybean trypsin inhibitor. The inhibitor was added at a protein concentration equal to that of trypsin. Incubation at room temperature was continued for an additional 10 min. The control membranes were treated in exactly the same way, except that trypsin was preincubated with trypsin inhibitor. After the treatment, the membranes were immediately utilized in the assays.

Preparation of depleted membranes and energy-transducing ATPase. The procedure used is a modification of that described by Bragg and Hòu (5) for preparation of the coupling factor (ATPase) from particulate fractions of *E. coli*. Sucrose gradient-purified cytoplasmic membranes were dialyzed for 6 h at 4°C against 1 mM tris(hydroxymethyl)aminomethane-hydrochloride buffer of pH 7.5, which con-

tained 0.5 mM EDTA, 0.5 mM dithiothreitol, and 8% sucrose. The dialyzed membranes were centrifuged for 3 h at 140,000 × *g* and 4°C. The sedimented, depleted membranes were suspended in the dialysis buffer at a protein concentration of about 2 mg/ml (at this step all fractions were stored overnight at 4°C). The supernatant fractions were concentrated to a protein concentration of about 2 mg/ml by using an Amicon ultrafiltration cell equipped with a PM10 membrane. These samples were used in reconstitution assays immediately after concentration and protein determination.

Reconstitution experiments. A volume of depleted membranes containing 0.45 mg of protein and a volume of concentrated supernatant (coupling factors) containing 0.9 mg of protein were added to the basic mixture of the energy-dependent transhydrogenase assay. This basic mixture contained 1.2 mg of bovine serum albumin, 12 μmol of $MgCl_2$, 45 μmol of tris(hydroxymethyl)aminomethane-hydrochloride (pH 7.8), and 3 μmol of Na_2S. The basic mixtures, with the samples (in a volume of 0.82 ml), were incubated for 20 min at room temperature; then the rest of the reaction components were added, and the assay was started as described previously (23).

RESULTS

Preparation and characteristics of cell wall membrane and cytoplasmic membrane fractions. *E. coli* disrupted in a variety of ways yields particulate fractions that are separable on sucrose gradients into cell wall membrane and cytoplasmic membrane (17, 19, 27). In this study, the methods of cell disruption and sucrose gradients were based on the same principles used by Schnaitman (27) and Osborn et al. (19), respectively. However, these methods were modified to obtain fractions competent in catalyzing the ATP-linked transhydrogenase reaction. Accordingly, *E. coli* K-12 C600, sensitive to colicins E1, E2, E3, and K, and *E. coli* K-12 C600 *tolA*, the tolerant derivative, were treated with colicin K and harvested, and particulate fractions were prepared by disrupting the bacteria in a French pressure cell (see above). The particulate fractions are separable on sucrose gradients into two peaks (data not shown). The low-density upper peak is the cytoplasmic membrane fraction. It contains the majority of the activities of the respiratory enzymes NADH oxidase, NADPH-NAD transhydrogenase (non-ATP dependent), and succinic dehydrogenase and the membrane-associated enzyme ATPase (Table 1). The high-density lower peak is the cell wall membrane fraction. It contains the majority of colicin E3 receptor activity (Table 1). Receptors of colicin E3 have been shown previously to be components of the cell wall membrane (25). Some cross-contamination existed between the two separated fractions. The cell wall fraction contained up to 22% of the total succinic dehydrogenase activity and only about 10% of the total ATPase and the total NADPH-NAD transhydrogenase. About 7% of the total colicin E3 receptor activity was retained in the cytoplasmic membrane. Treatment of bacterial cultures with colicin K had no apparent effect on the pattern of separation of the membranes. Colicin K treatment had no significant effect on the specific activities of respiratory enzymes or ATPase of the cytoplasmic membrane fraction (Table 1). These results are consistent with previous results obtained from particulate fractions (23).

Localization of ATP-linked transhydrogenase in cytoplasmic membrane fraction. ATP-linked transhydrogenase activity had previously been characterized in particulate fractions of *E. coli* (3, 8, 23, 28). The following experiments were conducted to ascertain that

TABLE 1. *Distribution of proteins and enzymes in cell wall and cytoplasmic membrane fractions*[a]

Parameter	Cytoplasmic membrane		Cell wall	
	U/mg of protein	% of total	U/mg of protein	% of total
Protein content		53		47
NADH oxidase	8.0	86	1.5	14
(untreated-treated)	(8.0-7.85)			
NADPH-NAD transhydrogenase	0.042	89	0.006	11
(untreated-treated)	(0.059-0.056)			
Succinic dehydrogenase	4.0	78	1.3	22
(untreated-treated)	(3.82-3.54)			
ATPase	0.223	91	0.025	9
(untreated-treated)	(0.194-0.19)			
Colicin E3 receptor titer	20	7.0	320	93

[a] Particulate fractions, prepared from *E. coli* K-12 C600, treated or untreated with colicin K, were separated on sucrose gradients into cell wall and cytoplasmic membrane fractions. Assays were carried out as described in reference 23. Data shown in parentheses represent compared activities of cytoplasmic membranes isolated from bacteria treated and untreated with colicin K.

the activity is conserved in the purified cytoplasmic membranes. The reduction of NADP to NADPH, linked to the oxidation of NADH (energy-linked transhydrogenase), was measured in the presence and absence of ATP. Figure 1 shows that ATP-linked transhydrogenase is catalyzed by particulate (cell wall plus cytoplasmic membrane) and cytoplasmic membrane fractions, but not by the cell wall fraction. The specific activity of the cytoplasmic membrane fraction is at least fourfold higher than the activity of the particulate fraction. Unlike the particulate fraction, the activity of the cytoplasmic membrane fraction is stable for prolonged periods of storage. The ATP-activated reaction of the cytoplasmic membrane fraction is inhibited by the energy uncouplers CCCP and DNP and by the ATPase inhibitor DCCD (data not shown). Cytoplasmic membrane and cell wall fractions catalyze a low-rate reduction of NADP to NADPH, which proceeds in the absence of ATP. This reaction is not significantly affected by CCCP, DNP, or DCCD (data not shown). Thus, it appears that the reaction, in the absence of ATP, does not require an energized state of membrane or a membrane-bound ATPase. Trypsin treatment of the membranes completely abolishes both the ATP-linked and unlinked reactions (data not shown). This indicates that protein components of the membrane essential for the reaction, in the presence or absence of ATP, are exposed on the surface of the membrane vesicles and are readily available for trypsin digestion.

Activities of membranes from bacteria treated or untreated with colicins. Colicins E1 and K adsorb to specific receptors located in the cell wall membrane of sensitive bacteria and affect biochemical targets, which are presumably located in the cytoplasmic membranes. The mechanism whereby the colicins, which are bound to the cell wall membrane, transmit their effect to the cytoplasmic membrane is largely unknown. Cytoplasmic membrane fractions of bacteria treated with colicin E1 or K are significantly inhibited in their ATP-linked transhydrogenase reaction (Fig. 2A and B). This result indicates that the majority of the colicins bound to the cell wall membrane are not required to maintain the colicin effect on the ATP-linked transhydrogenase reaction. The colicin effect is specific for colicins E1 and K; it is not observed in membranes of bacteria treated with colicin E2 or E3 (Fig. 2C and D).

Activities of membranes from tolerant bacteria and anaerobically grown, sensitive bacteria treated or untreated with colicin. Two stages are recognized in the process of killing by colicins (20). In the first stage, colicins adsorb to their specific receptors on the surface of the cells, but the cells do not suffer any physiological damage. In the second stage, the adsorbed colicins affect biochemical targets, which ultimately leads to cell death. Colicin-tolerant mutants bind colicins but are not killed by them. Treatment of C600 *tolA*, a colicin-tolerant mutant, with colicin K had no effect on the survival of these bacteria. The ATP-linked transhydrogenase reaction was not affected in the cytoplasmic membrane of these bacteria (Fig. 3A).

E. coli K-12 C600, grown under anaerobic conditions, were not killed by colicin K treatment. The treatment had no effect on the ATP-linked transhydrogenase of the isolated membranes (Fig. 3B). Exposure of the anaerobically grown bacteria to colicin E1 resulted in the killing of about 30% of the bacteria. Membranes from these bacteria showed some effect on the ATP-linked transhydrogenase of the cytoplasmic membranes (Fig. 3D). The anaerobi-

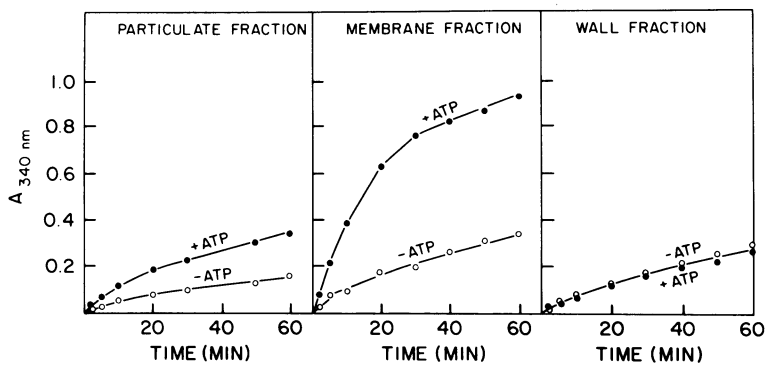


FIG. 1. *NADPH formation catalyzed by particulate fraction, cytoplasmic membrane fraction, and cell wall fraction from E. coli K-12 C600 in the presence and absence of ATP. Assay mixtures of 1-ml volumes received 0.45 mg of sample protein.*

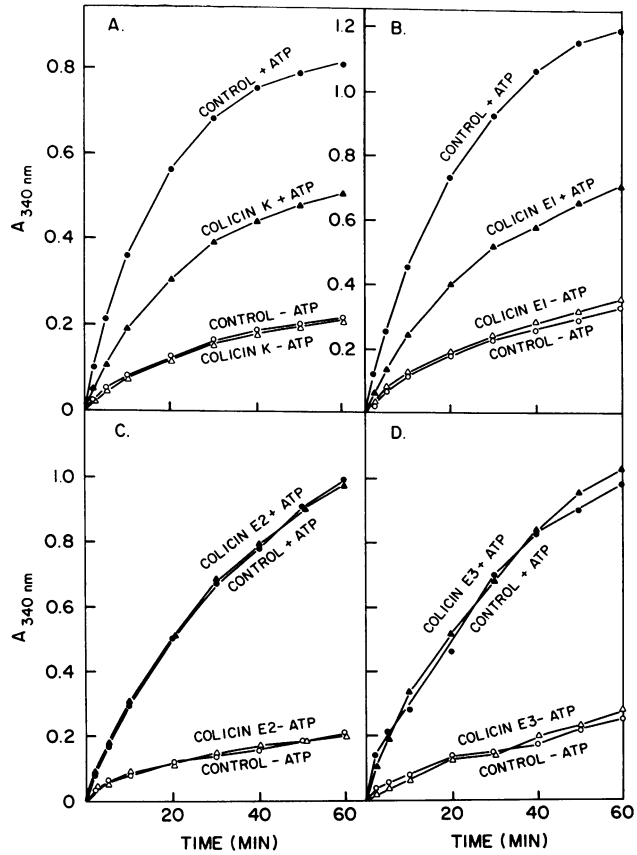


FIG. 2. *NADPH formation catalyzed by cytoplasmic membrane fractions prepared from control cells and cells treated with colicin. Cells treated (5 min at 37°C) with colicin K, E1, E2, or E3 gave survivals of 20, 1.2, 0.2, and 51%, respectively. Assay mixtures of 1-ml volumes received 0.45 mg of sample protein.*

cally grown, treated and untreated cultures were exposed to air for 2 h, with shaking. The bacteria in the untreated cultures doubled in number. In contrast, the majority of the bacteria of the treated cultures were killed. Cytoplasmic membranes prepared from the bacteria that were killed showed the colicin effect on ATP-linked transhydrogenase (Fig. 3C and E). These results suggest that the effect of colicins E1 and K on the membrane function is correlated with colicin lethality. The facts that colicins K and E1 were not effective in killing under anaerobic conditions and became effective after exposure to air of the treated bacteria indicate that these colicins were neither inactivated by some constituent of the anaerobic medium nor destroyed by some product of the anaerobic bacteria. Colicins E1 and K either do not adsorb to anaerobic bacteria or, under anaerobic conditions, the adsorbed colicins do not undergo the transition from their adsorption sites to their biochemical target sites.

Physical and biochemical treatments of cytoplasmic membranes. Several treatments were applied in an attempt to restore the membrane activity. The membranes from colicin-treated and untreated bacteria were subjected to one of the following treatments: ultrasonic waves (four pulses, 30 s each); repeated freezing and thawing (four times); incubation at 45°C for 10 min; or dialysis for 1 h against 0.05 M tris(hydroxymethyl)aminomethane, pH 7.8. None of these treatments restored the activity (data not shown). Trypsin treatment, which is known to rescue intact cells from colicin killing (18, 20, 30), cannot be used to rescue the membrane function, since it completely inhibits the reaction. Preliminary findings by Plate and Suit (Abstr. Annu. Meet Am. Soc. Microbiol. 1975, K186, p. 178) suggest that the reduction of intracellular cyclic adenosine 3',5'-monophosphate is an early consequence of colicin K action. However, when 2 mM cyclic adenosine 3',5'-monophosphate was added to the incubation mixtures, it caused a slight inhibition of the ATP-linked transhydrogenase of the mem-

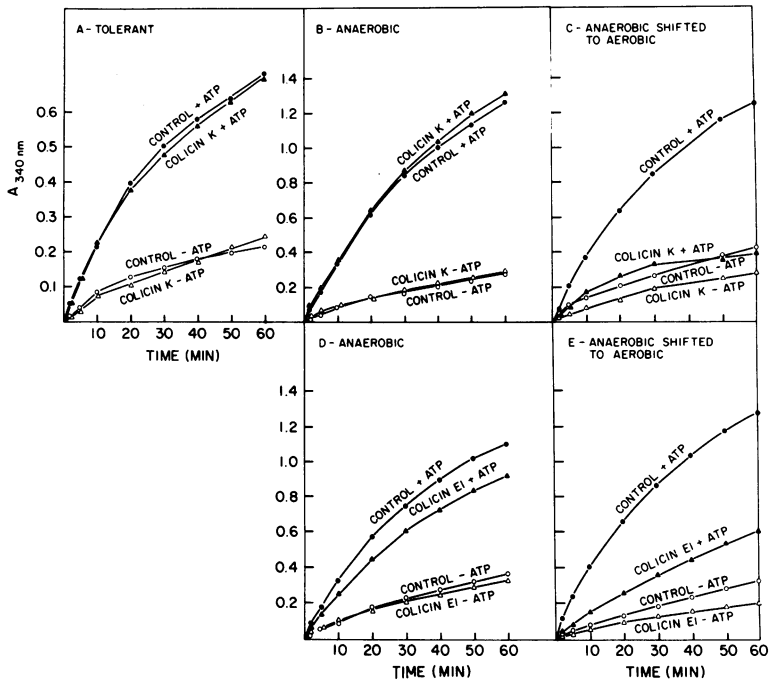


FIG. 3. *NADPH formation catalyzed by cytoplasmic membrane fractions prepared from bacteria treated or untreated with colicin K or E1 for 10 min at 37°C. Survival of colicin K-treated, anaerobic-sensitive and aerobic-tolerant cultures was 98 and 100%, respectively. The amount of colicin K used in treatment caused killing of 98% of the aerobically grown, sensitive bacteria. Survival of colicin E1-treated anaerobic bacteria was 70%. The amount of colicin E1 used in the treatment caused killing of more than 99% of the aerobically grown, sensitive bacteria.*

branes from the treated and untreated bacteria. Similar inhibition was obtained with adenosine 5'-monophosphate (data not shown).

ATP-linked transhydrogenase in dissociated and reconstituted cytoplasmic membranes. The ATP-linked transhydrogenase reaction is driven by an energized state of the membrane. This is evidenced by the finding that the reaction is inhibited by energy uncouplers (3, 8, 28). The energized state may be generated coupled to the hydrolysis of ATP. In this case, components of the membrane-bound ATPase complex catalyze the hydrolysis of ATP and the coupling of the energy released from ATP hydrolysis to the generation of the energized state. Alternatively, the energized state may be generated coupled to the oxidation of substrates by the respiratory chain. The presence of the structural components of the ATPase complex in the membrane appears to be necessary to maintain the energized state (2, 5, 22, 29). Thus, mutants that lost their ability to hydrolyze ATP while retaining the structural components of the ATPase complex maintain their capability to energize the membrane, via respiration (5). The ATPase-negative mutant, which was previously found to be sensitive to colicin K (21, 23), is an example of such mutants. Extraction of the ATPase complex from the membranes results in loss of both ATP-linked and respiratory-linked transhydrogenase (4). Both reactions are restored by readdition of the complex (2, 4, 5, 9). In the following experiments, the objective was to determine, through the use of reconstitution experiments, whether the colicin-induced defect is present in the solubilized, ATPase-containing portion of the cytoplasmic membrane or in the nonsolubilized portion of the cytoplasmic membrane (depleted membranes). Dialysis of cytoplasmic membranes, from bacteria that were treated or untreated with colicin E1, against a low-ionic-strength buffer, resulted in the release of a portion of the ATPase enzyme into the solubilized fraction (Table 2). Membranes that were depleted of the ATPase enzyme did not catalyze the ATP-linked transhydrogenase activity. This is evidenced from the finding that the presence of ATP in the reaction mixture does not stimulate the activity to levels higher than

those obtained in the absence of ATP (Fig. 4B). The addition of the solubilized fraction of the cytoplasmic membranes of either the treated or the untreated bacteria to the depleted membranes from control bacteria resulted in reconstitution of high levels of the ATP-linked transhydrogenase reaction (Fig. 4C). In contrast, when the solubilized fractions were added to the depleted membranes of treated bacteria, lower levels of the ATP-linked activity were obtained (Fig. 4D). Judging from initial rates, the solubilized fraction from treated bacteria is less efficient in reconstituting the activity. However, near-normal levels of activity were obtained when the reaction was allowed to reach steady-state levels. The low initial rates of activity obtained with the solubilized frac-

TABLE 2. *Distribution of protein and ATPase in fractions of dialyzed membranes*[a]

Membranes from:	Fraction	ATPase	
		U/mg of protein	Total U
Control	Depleted membrane	0.036	2.3
	Concentrated supernatant	0.134	4.82
E1 treated	Depleted membrane	0.034	1.98
	Concentrated supernatant	0.11	4.6

[a] Specific activities of ATPase of nondepleted membranes from control and treated bacteria were 0.21 and 0.20, respectively. Membranes were prepared from cells treated with colicin E1 as described in the legend to Fig. 4.

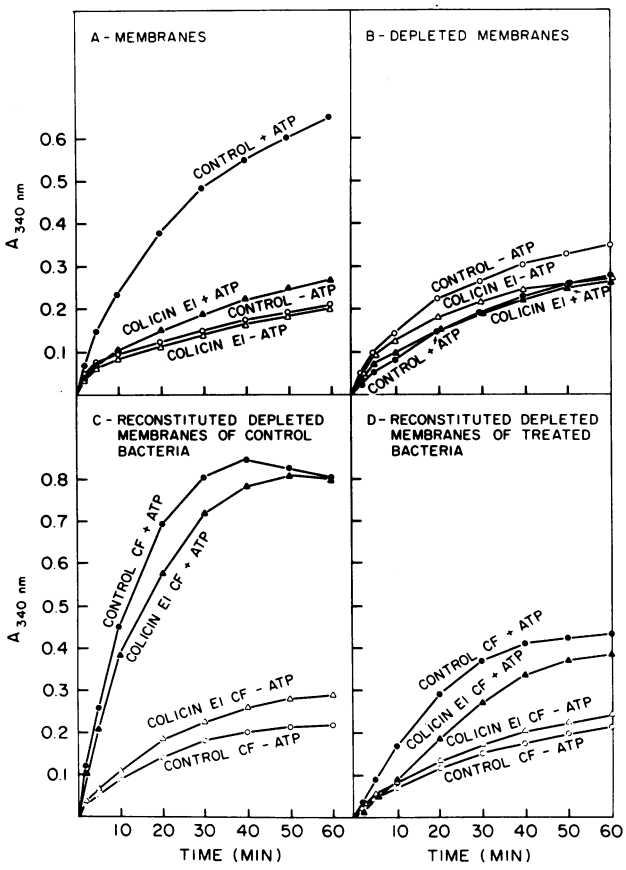


FIG. 4. *NADPH formation catalyzed by membranes, depleted membranes, and reconstituted membranes. The reaction was measured in (A) cytoplasmic membranes from control and colicin E1-treated bacteria; (B) depleted membranes from (A); (C) depleted membranes from control bacteria reconstituted with coupling factors from membranes of control or treated bacteria; (D) as in (C) except that depleted membranes were from treated bacteria. Assay mixtures of 1-ml volumes received 0.45 mg of membrane protein or of depleted membrane protein. Coupling factors were added at a protein concentration of 0.9 mg/ml of assay mixture. Cells treated with colicin E1 (5 min at 37°C) gave survivals of 0.1%.*

tion from membranes of treated bacteria may be due to its lower affinity to the membranes and/or a slightly lower specific activity of ATPase in this fraction. Similar results were obtained with reconstituted fractions from membranes of colicin K-treated bacteria. It should be pointed out that the specific activity of ATPase enzyme in the solubilized fractions of the dialyzed cytoplasmic membranes is lower than that of the native membranes (Table 2), and the recovery of this enzyme activity in the two fractions of the membrane (concentrated supernatant and depleted membrane) is only about 35%. The low recovery might be due to inactivation of the enzyme of the soluble fraction during the preparation procedures. It is conceivable that the presence of an inactive ATPase in the solubilized membrane fraction can interfere with the reconstitution of the activity by competing with active ATPase for binding sites on the depleted membranes. However, since the same solubilized membrane fraction is used to reconstitute the activity in depleted membranes of either the treated or the untreated bacteria, the presence of inactive ATPase is most likely to interfere with the reconstitution of the depleted membranes of either source. The important point here, illustrated in Fig. 4, is that the extent of the reconstituted activity appears to be determined by the source of the depleted cytoplasmic membrane fractions, not by the ATPase-containing solubilized membrane fractions.

DISCUSSION

The results reported here demonstrate that the ATP-linked transhydrogenase reaction is an energy-linked function of purified cytoplasmic membranes of *E. coli*. The reaction is inhibited by DCCD, CCCP, and DNP, confirming that the reaction requires a membrane-bound ATPase (5) and a functional energy-coupling system (3, 8, 28). The specific activity of the ATP-stimulated reaction of the cytoplasmic membrane is about fourfold higher than that of the total particulate fraction. The increase in specific activity is not totally due to removal of the outer membrane. It is possible that inhibitors of the reaction, proteases or lipases (26), are associated with the cell wall or retained in the periplasmic space of the particulate fraction and are eliminated during the purification of the cytoplasmic membrane. This view agrees with the observation that the activity of the cytoplasmic membrane fraction is stable for prolonged periods of storage (months) at −20°C, whereas the activity of the particulate fraction decays within a few weeks.

Both cytoplasmic membrane and cell wall catalyze a low-rate reduction of NADP to NADPH in the absence of ATP. In a previous report (23), I speculated that this reaction might represent the residual respiratory-linked transhydrogenase reaction. The energy for this reaction is derived from the oxidation of reduced substrates via the electron transport system, without the intervention of the ATP hydrolytic activity of ATPase enzyme. Fisher and Sanadi (8) showed that membrane particles prepared from anaerobic bacteria lacked the respiratory-linked transhydrogenase. Bragg and Hou (4) demonstrated that membrane particles depleted of energy-transducing ATPase lost the respiratory-linked reaction as well. The results shown in Fig. 3 and 4 demonstrate that cytoplasmic membranes of anaerobic bacteria, and cytoplasmic membranes depleted of energy-transducing ATPase, still catalyze the minus ATP reaction. These results exclude the possibility that the minus ATP reaction is a residual respiratory-linked transhydrogenase. The major evidence, though, against this possibility is the finding that the minus ATP reaction is not affected by energy uncouplers (unpublished data). Trypsin digestion abolishes both minus ATP and ATP-activated reactions. Therefore, protein components of these reactions are exposed to trypsin action.

One major result of this study is the finding that cytoplasmic membranes purified from colicin E1- or K-treated bacteria are impaired in their ability to catalyze the ATP-linked transhydrogenase reaction. The impaired property of the membrane is not dependent upon the presence of the majority of the bound colicin, since the majority of the cell wall membrane, together with the bound colicin, is removed from the cytoplasmic membrane during purification. However, the presence of about 7% of the total colicin E3 receptor activity in the ctyoplasmic membrane fraction (Table 1) indicates that the cytoplasmic membrane is partially contaminated with components of the cell wall membrane. The presence of the cell wall membrane components in the cytoplasmic membrane fraction may be due to a nonspecific cross-contamination between the two separated peaks of the sucrose gradients. Alternatively, the cell wall components may represent regions of the cell wall membrane that are in close association with the cytoplasmic membrane. It is conceivable that such regions might contain colicin receptors that are critical for the expression of colicin action in the cytoplasmic membrane.

The activity of the ATPase enzyme and respiratory enzymes is not affected in membranes

from colicin K-treated bacteria. Evidently, a gross conformational change of the membrane that affects these activities does not occur. These results confirm my earlier finding with particulate fractions (23) and support the idea that the membranes from colicin E1- or K-treated bacteria are impaired in some components of their energy-coupling system (6, 14, 21, 23). Since colicins E1 and K inhibit ATP-linked transhydrogenase of isolated membranes (23) and active transport processes of intact cells (13, 16, 21) and membrane vesicles (1), it is likely that the colicins inhibit these energy-linked functions through their action on components of the energy-coupling system of the membranes common to these functions. Energy-dependent transhydrogenase reactions are amenable to resolution and reconstitution studies that should lead to the identification of the defective components. Bragg and Hou (4) showed that removal of an energy-transducing ATPase enzyme complex from particles of *E. coli* results in loss of both respiratory- and ATP-linked transhydrogenase, which are restored upon readdition of this enzyme complex. The results of depletion and reconstitution experiments obtained in this study show that the energy-transducing ATPase complex from colicin E1- or K-treated bacteria are nearly as effective as the enzyme complex of normal bacteria in restoring the ATP-linked transhydrogenase to depleted membranes. Therefore, the complex from treated bacteria is capable of energizing the membranes. The finding that the level of the restored activity in depleted membranes from colicin-treated bacteria is markedly lower than the restored activity in depleted membranes from untreated bacteria suggests that the colicin-caused defect is in the depleted membrane. The depleted membranes of colicin-treated bacteria are impaired in their ability to bind the energy-transducing ATPase and/or the ATPase is bound to a membrane matrix that is ineffective in energy transduction.

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