ISOLATION OF *ESCHERICHIA COLI* MUTANTS WHICH ARE RESISTANT TO AN INHIBITOR OF H^+-ATPase, TRIBUTYLTIN AND ALSO TO UNCOUPLERS OF OXIDATIVE PHOSPHORYLATION

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Received 14 September 1981; revision received 10 November 1981

1. Introduction

ATP formation by oxidative phosphorylation or photophosphorylation is observed in bacterial, mitochondrial and chloroplast membranes [1–3]. According to Mitchell's chemiosmotic hypothesis, the transmembrane gradient of electrochemical potential of H^+ formed by the translocation of proton across the membranes is the motive force for ATP formation [4]. The proton-translocating ATPase (H^+-ATPase) is thought to play the central role in ATP synthesis [5].

We tried to isolate mutants of *Escherichia coli* which were resistant to an inhibitor of H^+-ATPase, tributyltin, in order to understand the ATP formation in biological energy-transducing membranes. Here, we report that almost all of them are also resistant to uncouplers of oxidative phosphorylation, carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) and/or pentachlorophenol (PCP). We also present evidence indicating that uncouplers interact with H^+-ATPase.

2. Materials and methods

2.1. *Bacteria*

Strain KH434 (*gal,thy*), which was derived from strain W3350, was provided by Dr T. Horiuchi. Strain KM135 (HfrC,*ilvA,met,rel,tonA*$_{22}$,T2^r,*bglB*$^-$*R*$^-$, *tna*) was provided by Mr K. Joh. IM5 (KM135 *met*$^+$) was constructed from KM135 by P1-transduction. KL728 which harbors the F-prime factor, F111, was provided by Dr B. Bachmann.

Abbreviations: CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; PCP, pentachlorophenol; EMS, ethyl methanesulfonate; DCCD, *N,N'*-dicyclohexylcarbodiimide

2.2. *Medium*

M63 medium [6] was used as the minimal medium. Sodium succinate (40 mM) or 0.4% (w/v) glucose was used as the carbon source. Thymine was added at 10 μg/ml final conc. when KH434 was grown. L-Broth supplemented with 10 μg thymine/ml was used as the rich medium.

2.3. *Chemicals*

CCCP was purchased from Sigma. Tributyltin, *N,N'*-dicyclohexylcarbodiimide (DCCD), sodium azide and ethyl methanesulfonate (EMS) were purchased from Wako Chemicals. PCP was provided by Dr T. Higuchi.

2.4. *Isolation of tributyltin-resistant mutants*

Tributyltin (4×10^{-3}% (v/v)) was used for the isolation of mutants. About 5×10^8 cells were spread on a plate containing succinate, thymine and tributyltin, and incubated at 30°C.

2.5. *Test of minimal inhibitory concentration*

Cells were grown to log phase in the rich medium and washed with M63 medium. About 10^6 cells were put on each plate which contained succinate, thymine and an inhibitor. Inhibitors were 25 μM, 30 μM, 40 μM, 50 μM and 60 μM for CCCP; 75 μM, 100 μM, 150 μM, 200 μM and 300 μM for PCP; 50 μM, 100 μM, 200 μM and 300 μM for sodium azide; 1.5 mM, 3 mM and 5 mM for DCCD.

Published by Elsevier/North-Holland Biomedical Press
00145793/81/0000–0000/$02.75

3. Results

3.1. *Isolation of mutants which are resistant to tributyltin*

At 4×10^{-3}% (v/v) of the concentration of tributyltin used for the isolation of mutants, the growth of wild-type strain KH434 was inhibited when succinate was used as the carbon source but not when glucose was used (not shown). This observation suggests that tributyltin inhibits metabolically ATP formation by oxidative phosphorylation which occurs after the tricarboxylic acid cycle. After cells were spread on plates containing tributyltin, the incubation was performed at 30°C for 3 days. Colonies which appeared were picked up and purified twice on the selective plate. Four spontaneous resistant mutants were isolated and an additional 17 resistant mutants were isolated after EMS-mutagenesis. The minimal inhibitory concentration for tributyltin of each strain was indicated in table 1. The minimal inhibitory concentration of wild-type strain KH434 was 1×10^{-3}% and those of mutant strains varied 2×10^{-3}–4×10^{-3}%. A spontaneous mutant strain SM422 (*ttr-1*) and a EMS-induced mutant strain SE2110 (*ttr-6*) were grown in a liquid medium containing tributyltin to confirm the resistance. The growth of strain KH434 was completely inhibited in the presence of 5×10^{-4}% (v/v) of tributyltin when succinate was used as the carbon source but not when glucose was used (fig.1). This observation suggests that tributyltin inhibits ATP formation by oxidative phosphorylation. Strain SM422 and SE2110 could grow at that concentration of tributyltin (fig.1). Similar observations were obtained when the concentrations of tributyltin were 2.5×10^{-4}% and 1×10^{-3}% (not shown).

3.2. *Co-drug resistance*

We have isolated the uncoupler-resistant mutants which were also resistant to some of H^+-ATPase inhibitors, tributyltin, sodium azide and DCCD (in prepa-

Table 1
Minimal inhibitory concentration for inhibitors of H^+-ATPase and uncouplers

Strain	Mutated allele	Tributyltin ($\times 10^{-3}$%)	Sodium azide (μM)	DCCD (mM)	CCCP (μM)	PCP (μM)
KH434	wild	1	50	1.5	25	75
SM422	*ttr-1*	3	300	5	30	200
SM425	*ttr-2*	3	s	s	s	150
SM434	*ttr-3*	3	300	5	40	150
SM311	*ttr-4*	2	300	5	s	200
SE211	*ttr-5*	3	s	s	s	150
SE2110	*ttr-6*	4	s	>5	30	150
SE2111	*ttr-7*	4	s	5	40	200
SE2113	*ttr-8*	4	300	s	30	300
SE2118	*ttr-9*	3	300	5	s	200
SE2210	*ttr-10*	4	200	>5	s	200
SE2215	*ttr-11*	3	300	>5	s	300
SE225	*ttr-12*	3	150	s	30	300
SE231	*ttr-13*	4	150	s	30	200
SE232	*ttr-14*	3	150	s	s	s
SE233	*ttr-15*	4	s	s	s	200
SE238	*ttr-16*	3	s	s	s	150
SE2310	*ttr-17*	3	s	5	s	300
SE2456	*ttr-18*	1	300	s	30	s
SE246	*ttr-19*	3	150	5	s	s
SE247	*ttr-20*	4	150	s	s	300
SE2466	*ttr-21*	3	150	s	40	200

's' means that the growth of each mutant strain was inhibited on plate containing 50 μM sodium azide, 1.5 mM DCCD, 25 μM CCCP or 75 μM PCP. SE2456 (*ttr-18*) was isolated on the plate containing tributyltin. However, the growth was inhibited on the plate containing 1×10^{-3}% tributyltin, when the minimal inhibitory concentration was tested. Succinate was used as the carbon source

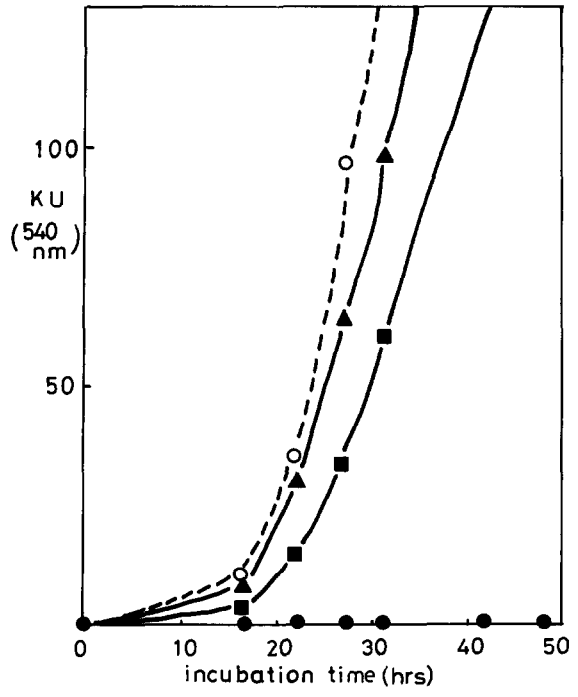

Fig.1. Resistances to tributyltin of strains SM422 and SE2110. Cells were grown to 3 × 10^8/ml in the rich medium, washed and diluted to 3 × 10^6/ml in M63 medium containing 5 × 10^{-4}% (v/v) tributyltin, which was supplemented with glucose or succinate. Incubations were performed at 30°C and turbidity in Klett units (540 nm) was measured: KH434, glucose (○); KH434, succinate (●); SM422, succinate (■); SE2110, succinate (▲). Strains SM422 and SE2110 showed good growths in the presence of tributyltin when glucose was used as the carbon source (not shown).

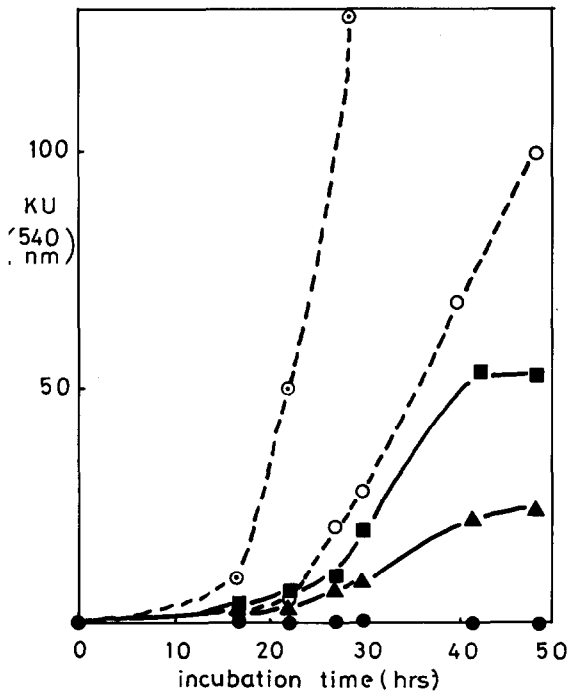

Fig.2. Resistances to uncouplers of strain SM422. Cells were grown to 3 × 10^8/ml in the rich medium, washed and diluted to 3 × 10^6/ml in M63 medium containing 100 μM PCP or 30 μM CCCP. Glucose or succinate was used as the carbon source. Incubations were performed at 30°C and turbidity in Klett units (540 nm) was measured: KH434, glucose, PCP (⊙); KH434, glucose, CCCP (○); KH434, succinate, PCP or CCCP (●); SM422, succinate, PCP (■); SM422, succinate CCCP (▲). Strain SM422 showed good growth in the presence of an inhibitor when glucose was used as the carbon source (not shown).

ration). Therefore, we tested whether the tributyltin-resistant mutants were resistant to other H^+-ATPase inhibitors or uncouplers. Table 1 indicates the minimal inhibitory concentrations of mutants for inhibitors. SE232 (*ttr-14*) and SE246 (*ttr-19*) are resistant to not only tributyltin but also sodium azide. The remaining 19 mutants are resistant to not only some of H^+-ATPase inhibitors, tributyltin, sodium azide and DCCD but also uncouplers, CCCP and/or PCP. A typical mutant strain SM422 was grown in a liquid medium containing each uncoupler in order to confirm the co-drug resistance. Strain SM422 was able to grow fairly in the presence of 100 μM PCP or 30 μM CCCP which inhibited the growth of wild-type strain KH434 completely (fig.2).

CCCP and PCP are uncouplers of oxidative phosphorylation and tributyltin, sodium azide and DCCD are H^+-ATPase inhibitors [5]. The co-drug resistances observed in these mutants, which indicate that the resistances to the H^+-ATPase inhibitors affect the resistances to uncouplers, suggest that the component(s) of H^+-ATPase complex is affected by uncouplers.

3.3. *Co-transduction of* ttr-1 *and* ttr-6 *with* ilvA *genes*

Since it is known that tributyltin, sodium azide and DCCD are inhibitors of H^+-ATPase [5], we suppose that each mutant strain carries a mutation in protein subunit(s) of H^+-ATPase. *unc* Genes coding for protein subunits of H^+-ATPase are mapped at 83.5 min on the chromosome of *E. coli* and can be co-transduced with *ilv* genes by P1 phage [7]. We did P1-transduction experiments to learn whether typical mutant strain, SM422 (*ttr-1*) and SE2110 (*ttr-6*) car-

ried mutations in protein subunit(s) of H^+-ATPase. Strain IM5 was used as the recipient strain and *ilv*$^+$ transductants were selected. The experiments indicated that *ttr-1* allele linked to *ilvA* gene with the frequency of 30% (12 out of 40 tested) and that *ttr-6* allele did with 11% (6 out of 57 tested). The experiments of transduction indicated that the tributyltin-resistant mutant strains carried mutations in protein subunit(s) of H^+-ATPase.

3.4. *Isolation of temperature-sensitive* unc *mutants*

The linkages with *ilvA* gene and the resistances to other inhibitors of H^+-ATPase, sodium azide and/or DCCD indicate that the tributyltin-resistant mutant strains carry mutations in protein subunit(s) of H^+-ATPase. If it is true, some of them should present the *unc* phenotypes, which means that cells can grow in the presence of glucose as the carbon source but not in the presence of succinate. Four of 21 mutant strains, SM434 (*ttr-3*), SM311 (*ttr-4*), SE2215 (*ttr-11*) and SE2456 (*ttr-18*), could grow in the presence of glucose as the carbon source but not in the presence of succinate when the experiments were performed in the absence of each inhibitor at 42°C. A spontaneous mutant strain SM434 (*ttr-3*) of these temperature-sensitive *unc* mutant strains was examined more extensively. Fig.3 indicates that strain SM434 is a temperature-sensitive *unc* mutant. It was confirmed by the mating experiment that strain SM434 was an *unc* mutant strain. When strain SM434 was mated with strain KL728 harboring F111 containing *unc* region of the chromosome [8], the F111-introduced

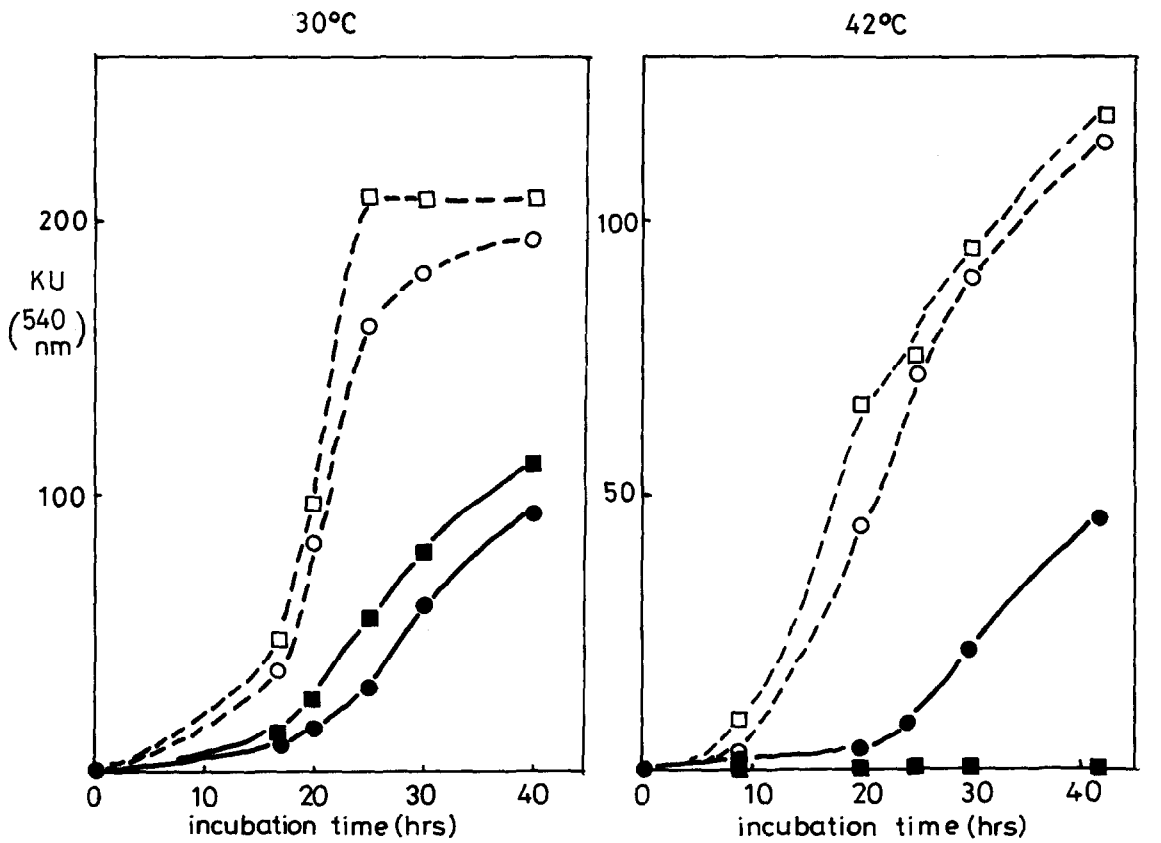

Fig.3. Temperature-sensitive *unc* phenotype of strain SM434. Cells were grown to 3 × 10^8/ml in the rich medium, washed and diluted to 3 × 10^6/ml in M63 medium. Glucose or succinate was used as the carbon source. Incubations were performed at 30°C or 42°C and turbidity in Klett units (540 nm) was measured: KH434, glucose (○); KH434, succinate (●); SM434, glucose (□); SM434, succinate (■). The approximate number of viable cells counted after 30 h was as follows at 30°C: KH434, succinate 2.0 × 10^8/ml; SM434, succinate 3.0 × 10^8/ml, at 42°C, KH434, glucose 1.0 × 10^9/ml; KH434, succinate 5.8 × 10^8/ml; SM434, glucose 9.5 × 10^8/ml; SM434, succinate 1.4 × 10^6/ml.

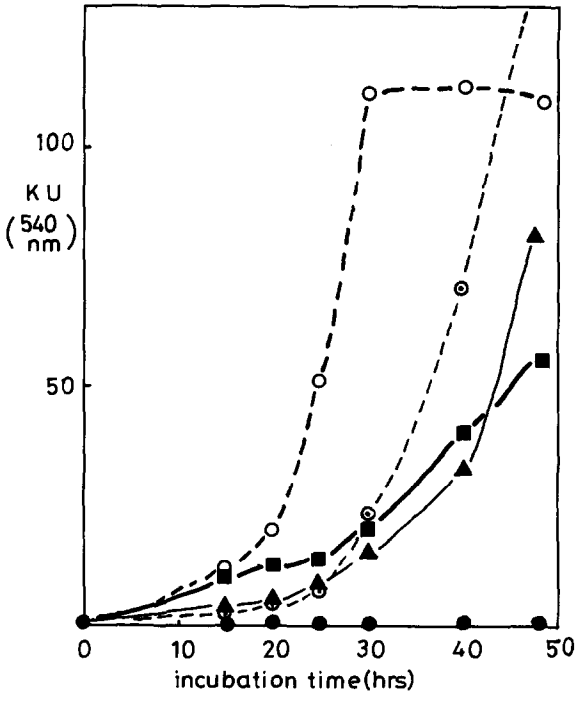

Fig.4. Resistances to tributyltin and PCP of strain SM434. Cells were grown to 3 X 10^8/ml in the rich medium, washed and diluted to 3 X 10^6/ml in M63 medium containing 2.5 X 10^{-4}% tributyltin or 100 μM PCP. Glucose or succinate was used as the carbon source. KH434, glucose, tributyltin (○); KH434, glucose, PCP (⊙); KH434, succinate, tributyltin or PCP (●); SM434, succinate, tributyltin (■); SM434, succinate, PCP (▲). Incubations were performed at 30°C. Strain SM434 showed good growth in the presence of an inhibitor when glucose was used as the carbon source.

SM434 could grow on the succinate plate at 42°C. This mating experiment also indicates that wild type allele is dominant to the mutated allele, *ttr-3*.

Strain SM434 is resistant to tributyltin, sodium azide, DCCD, CCCP and PCP (table 1). It is shown in a liquid medium that strain SM434 can grow in the presence of tributyltin or PCP (fig.4). We examined whether the temperature-sensitive *unc* phenotype and the resistances to 5 inhibitors were induced by a single mutation. When 20 spontaneous *unc*$^+$ revertants were picked up on the succinate plate at 42°C, 3 revertants of them simultaneously reverted to the same sensitivities to 5 inhibitors as wild-type strain KH434. Eight of the remaining 17 revertants did not change their resistances. The remaining 9 revertants reverted the sensitivities to some of 5 inhibitors. The details are indicated in table 2. The reversion test indicates that

Table 2
Reversion test of strain SM434

Tributyltin	Sodium azide	DCCD	CCCP	PCP	No. of revertants
s	s	s	s	s	3
r	r	r	r	r	8
r	r	r	s	s	2
r	r	s	s	s	1
r	r	s	r	r	2
r	s	s	s	s	1
r	s	s	s	r	1
s	r	s	s	s	1
s	s	r	r	s	1

's' means that the growth of each revertant strain was inhibited on plate containing 1 X 10^{-3}% tributyltin, 50 μM sodium azide, 1.5 mM DCCD, 25 μM CCCP or 75 μM PCP which inhibited the growth of wild type strain KH434; 'r' means that each revertant strain can grow in the presence of the concentration of the inhibitor. Twenty *unc*$^+$ revertants which were picked up on succinate plate at 42°C, were tested. Succinate was used as the carbon source

the temperature-sensitive *unc* phenotype and the resistances to 5 inhibitors are induced by a single mutation, because true revertants have been isolated.

4. Discussion

We isolated 4 spontaneous mutants and 17 EMS-induced mutants which were resistant to tributyltin. Two of 21 tributyltin-resistant mutants were also resistant to sodium azide which is an inhibitor of H^+-ATPase. The remaining 19 mutants were resistant to not only either or both of inhibitors of H^+-ATPase, sodium azide and DCCD but also uncouplers of oxidative phosphorylation, CCCP and/or PCP.

We conclude that these mutant strains carry mutations in protein subunit(s) of H^+-ATPase and that uncouplers, CCCP and PCP interact with H^+-ATPase, according to the following reasons:

(i) High correlations between the resistances to the inhibitors of H^+-ATPase and those to uncouplers are observed in the mutant strains;
(ii) The 3 mutated alleles examined are mapped in the region which is expected for *unc* genes;
(iii) Some mutant strains (4 out of 21) present *unc* phenotypes at 42°C;
(iv) When a temperature-sensitive *unc* mutant strain SM434 (*ttr-3*) was examined more extensively,

spontaneous true revertants were isolated. This reversion test indicates that the temperature-sensitive *unc* phenotype and the resistances to tributyltin, sodium azide, DCCD, CCCP and PCP are induced by a single mutation.

Here, we cannot rule out a possibility that a mutation in H^+-ATPase affects the transport of inhibitors. However, the partial revertants (9 out of 20 tested) which reverted sensitivities to some of 5 inhibitors, were isolated by a single step reversion from SM434 (table 2). It is likely from this observation that the substitution of a different amino acid at the mutated site or an amino acid at the other site in H^+-ATPase affects the binding of each inhibitor with H^+-ATPase.

Our conclusion is compatible with [9,10] that a CCCP-resistant mutant strain of *Bacillus megaterium* had the altered H^+-ATPase activity and [11] that azide–nitrophenol-binding protein is a protein subunit of H^+-ATPase in beef-heart mitochondria. Also, the conclusion is confirmed by the observations that CCCP- or PCP-resistant mutant strains are also resistant to some of H^+-ATPase inhibitors, tributyltin, sodium azide and DCCD and that the mutated alleles are mapped in a similar region as *unc* genes (in preparation).

Acknowledgements

We thank Professor M. Nishimura for reading the manuscript and Professors T. Omura and M. Sekiguchi for discussions. We also thank Dr T. Higuchi for providing PCP, and Drs B. Bachmann and T. Horiuchi and Mr K. Joh for providing bacterial strains.

References

[1] Cox, G. B. and Gibson, F. (1974) Biochim. Biophy. Acta 346, 1–25.
[2] Kovac, L. (1974) Biochim. Biophy. Acta 346, 101–135.
[3] Nelson, N. (1976) Biochim. Biophy. Acta 456, 314–338.
[4] Mitchell, P. (1966) Biol. Rev. 41, 445–502.
[5] Kagawa. Y., Sone, N., Hirata, H. and Yoshida, M. (1979) J. Bioenerg. Biomembr. 11, 39–77.
[6] Miller, J. H. (1972) in: Experiments in Molecular Genetics, p. 431, Cold Spring Harbor Lab., New York.
[7] Downie, J. A., Gibson, F. and Cox, G. B. (1979) Annu. Rev. Biochem. 48, 103–131.
[8] Low, K. B. (1972) Bact. Rev. 36, 587–607.
[9] Decker, S. J. and Lang, D. R. (1977) J. Biol. Chem. 252, 5936–5938.
[10] Decker, S. J. and Lang, D. R. (1978) J. Biol. Chem. 253, 6738–6743.
[11] Berden, J. A. and Henneke, M. A. C. (1981) FEBS Lett. 126, 211–214.