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EXTRINSIC AND INTRINSIC FACTORS THAT INFLUENCE INACTIVATION AND PURIFICATION OF THE UNSTABLE ADENOSINE TRIPHOSPHATASE SOLUBILIZED FROM MEMBRANES OF AN *ESCHERICHIA COLI* K 12 STRAIN

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Summary

Washing with EDTA at pH 9.0 was an obligatory step to solubilize completely the adenosinetriphosphatase (ATPase) (ATP phosphohydrolase, EC 3.6.1.3) from membranes of *Escherichia coli* K 12 strain 414. After solubilization, the enzyme was highly unstable (half-life at 4°C < 4 h) and this instability hindered its purification. Alkaline pH and EDTA were among the extrinsic factors responsible for the instability of the enzyme because the half-life of the ATPase was increased to >30 days by lowering its pH to 7.5 and adding 3—5 mM Mg^{2+}. Inactivation of the ATPase of *E. coli* K 12 strain 414 was associated with the appearance of components of higher and lower mobility than an ATPase band of relative mobility 0.22 ± 0.02 (Tris/glycine system, pH 8.5 ± 0.2).

The ATPase of *E. coli* K 12 strain 414 was purified and, under conditions of maximal stability, it yielded an homogeneous protein (by gel electrophoresis in Tris/acetate buffers, pH 7.5) which had a basal specific activity of 20 μmol substrate transformed · min^{-1} · mg $protein^{-1}$ and was stimulated by trypsin to 50 μmol · min^{-1} · mg^{-1}. The nature and/or pH of the buffer electrophoretic system strongly influenced the protein profile of purified ATPase. The Tris/glycine system induced the appearance of several components as a likely result of dissociation of purified ATPase. This increase in the number of bands induced by external factors is termed extrinsic heterogeneity.

Electrophoresis in sodium dodecyl sulphate (Tris/glycine, pH 8.5) revealed the presence of five subunits: α (mol. wt; 54 000 ± 2000); β (48 000 ± 3000), γ (small proportion: 30 000 ± 3000), δ (21 000 ± 2000) and ε (13 000), together with a component x (mol. wt. about 120 000) whose significance as a true subunit is questionable. By dodecyl sulphate electrophoresis in Tris/acetate (pH 7.5) we detected only four subunits (α, β, γ and ε) and component x. In this system, the molecular weights of the major subunits (α, 66 000; β, 60 000)

were slightly higher. These results represent small molecular differences with regard to ATPases from other *E. coli* strains and are discussed in relationship to the stability of the ATPase from *E. coli* K 12 strain 414.

Introduction

The solubilization and purification of the membrane-bound adenosinetriphosphatase (ATPase or BF_1 factor) (ATP phosphohydrolase, EC 3.6.1.3) from different strains of *Escherichia coli* has been the subject of several recent reports from various laboratories [1—8]. While studying the ATPase from one strain of *E. coli* K 12, Carreira et al. [9] found that the enzyme was completely solubilized by membrane washing with EDTA buffers of alkaline pH values. But the soluble enzyme was highly unstable and this instability hindered its purification. The rapid inactivation was accompanied by the appearance of components of higher mobility in gel electrophoresis than the active enzyme. On the other hand, attempts to solubilize the enzyme by membrane washing with low ionic strength buffers without Mg^{2+}, effective with ATPases from other *E. coli* strains [1,4], gave very low percentages of ATPase activity of even higher instability (ref. 9 and Azocar, O., unpublished results).

We thought it was of interest to study the extrinsic factors responsible for such a high instability of the ATPase of the *E. coli* strain studied by us. After solving, at least in part, this problem, we purified the enzyme and studied some of its molecular properties in an attempt to define the intrinsic factors responsible for the instability. The present work has provided some basis to understand the extreme lability of the ATPase of *E. coli* K 12 strain 414 although the molecular bases for it are not yet clearly understood.

Materials and Methods

The microorganism used (*E. coli* K 12, strain 414, *Hfr*$^-$, *thr*$^-$), its growth conditions and the isolation of a fraction enriched in inner membranes (membrane "ghosts") have been described in detail elsewhere [1,10].

ATPase extraction and measurement of ATPase activity. ATPase was extracted by suspending the membranes (1 mg protein/ml) in 3 mM EDTA/30 mM Tris · HCl (pH 9.0) as described by Carreira et al. [9]. As source of soluble ATPase, the extract was used as obtained or was filtered through an Amicon apparatus with a Diaflo PM-10 membrane (1—1.5 kg/cm^2) to eliminate EDTA or change the buffer composition as detailed under Results; the filtration was repeated 3—4 times using each time the same original volume. In some instances, the ATPase was extracted by the sequential washing of the membranes with Tris · HCl buffers (pH 7.5) of decreasing molarities [9]. These soluble enzymes were stored at 4° C for different periods of time.

Basal and trypsin-stimulated ATPase activity of fresh or stored preparations was measured colorimetrically [11] using 8 mM ATP/4 mM $MgCl_2$ essentially as described by Carreira and Muñoz [12]. This substrate concentration was 7-fold higher than the $S_{0.5}$ of the enzyme [12]. Protein content was determined by the method of Lowry et al. [13].

Analytical and semi-preparative gel electrophoreses. The electrophoresis at alkaline pH (8.5 ± 0.2) with the Tris/glycine buffer system was carried out as reported [14,15]. For electrophoresis at neutral pH (7.5), gels were prepared in all cases as described by Williams and Reisfeld [16]. The following buffers (pH 7.5) were used in both upper and lower chambers: 14.6 mM Tris/30 mM 5,5'-diethylbarbituric acid; 7.3 mM Tris/15 mM, 5,5'-diethylbarbituric acid; 3.6 mM NaH_2PO_4/31 mM Na_2HPO_4; 1.8 mM NaH_2PO_4/15.5 mM Na_2HPO_4; 22 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulphonic acid (HEPES)/3 mM imidazole [7]; 40 mM Tris/20 mM sodium acetate titrated with acetic acid to reach the pH 7.5 [17].

Sodium dodecyl sulphate electrophoresis was run in the same conditions but adding 0.1% (w/v) sodium dodecyl sulphate. Samples were treated (85°C, 5 min) with 4 mg dodecyl sulphate/mg protein.

Proteins were stained with Coomassie Blue R-250 [17] or following the rapid procedure of Dietzel et al. [18] with Coomassie Blue G-250. ATPase activity was visualized in the gels by adapting the histochemical method described previously [19].

Analytical gels of 11 × 0.5 cm and two acrylamide concentrations (7% (w/v) acrylamide/0.17% (w/v) *N,N'*-methylenebisacrylamide or 5% acrylamide/0.12% bisacrylamide) were used. Semipreparative electrophoresis was performed with 7% acrylamide gels of 21 × 2 cm, run overnight with the Tris/acetate buffer system. Proteins were stained by the Coomassie G-250 method on a thin (approx. 3 mm) longitudinal slice and once the mobility of the ATPase was measured, the protein was eluted with 30 mM Tris · HCl (pH 7.5) from the transversal slices of the superimposed zones.

All electrophoreses were run at 4°C in chambers designed by the Instrumentation Department of this Center and at a constant voltages that did not increase current intensity over the range 4—5 mA/gel. Bromophenol blue was used as tracking dye. Gels were scanned at 575 nm in a Gilford spectrophotometer 2400 equipped with the 2410 S linear transport system.

Gel chromatography. Sephadex G-200 filtration was performed on a Pharmacia K-50 column (80 × 5 cm), total volume of about 1700 ml and V_o (determined with blue dextran) of 480 ml. Sepharose 4B experiments were run on a Pharmacia K 25 column (84 × 2.5 cm), V_o = 198 ml. All these experiments were carried out at room temperature. Protein was monitored at 206 and 278 nm with the LKB Uvicord III.

Chemicals. Acrylamide (electrophoresis grade) and *N,N'*-methylenebisacrylamide were from Eastman Kodak Chemicals; *N,N,N',N'*-tetramethylethylendiamine was obtained from Fluka. Sodium dodecyl sulphate (sodium lauryl sulphate, specially pure) was from British Drug Houses and dithiothreitol from Calbiochem. Adenosine triphosphate, disodium salt was purchased from P-L Biochemicals. The following proteins were used as molecular weight standards: bovine serum albumin (mol. wt. 66 000) and egg albumin (43 000) were from Sigma Chem. Co.; lysozyme (14 000) and trypsin (23 000) were from Calbiochem, and lactate dehydrogenase for analytical purposes (35 000) was from Boehringer Mannheim GmbH. All other reagents of the high quality commercially available were obtained from Merck, (Darmstadt, West Germany).

Results

Extrinsic factors that effect inactivation of the soluble ATPase from E. coli K 12 strain 414. Protective effect of neutral pH and Mg^{2+}

The rapid inactivation of the ATPase after solubilization from the membranes prevented further work. The results on the time course of inactivation of the EDTA(pH 9)-soluble enzyme as reported by Carreira et al. [9] suggested that the procedure of solubilization was harmful to the activity. The definition of the role of each extrinsic factor in inactivating the ATPase seemed to be necessary for enzyme purification.

The possible damaging effect of EDTA was demonstrated by an increase in stability of ATPase after titrating the EDTA of the soluble enzyme fraction with Mg^{2+}. The effect of pH on the stability of the soluble enzyme was tested in samples stored without EDTA at three different pH values. Fig. 1 illustrates the ATPase patterns in the Tris/glycine electrophoretic system (see Materials and Methods) of 3-days-old samples. A minimum of three to four bands showed positive ATPase staining. Also shown are the half-life values for the enzyme activity at these pH values. The alkaline range induced a more rapid inactivation of the ATPase. The inactivation was paralleled by the disappearance of the slowest moving band, (relative mobility 0.22 ± 0.02) with a concomitant increase of the second predominant band (relative mobility 0.31 ± 0.02). One of the fast moving components (0.60 ± 0.02) showed a relative increase in its intensity following inactivation.

The protective effect of Mg^{2+} on the stability of the enzyme is emphasized in Table I. An apparent optimum was attained at 3—5 mM Mg^{2+}. The effect of Mg^{2+} on the protein profile was analyzed by gel electrophoresis of the unfractionated soluble preparation in Tris/glycine (pH 8.5) as shown in Fig. 2. In spite of the complex pattern of this crude soluble enzyme fraction, the effect was clearly seen at two different levels with regard to *E. coli* ATPase. First Mg^{2+}, in good correlation with the high activity, maintained the ATPase predominantly as a band of relative mobility 0.22 ± 0.02 (see also Fig. 1). Second, the bands of relative mobility 0.13 and 0.60 present in an old enzyme preparation were virtually absent when Mg^{2+} was added.

The effect of ionic strength was examined by adding increasing amounts of KCl to the soluble fraction equilibrated at pH 7.5 (30 mM Tris · HCl). By adding 175 mM KCl, the half-life of ATPase activity was decreased to 4 days as compared with 13 days for the Tris buffer alone (see Fig. 1).

Purification of the ATPase from E. coli K 12, strain 414 by molecular sieving

Taking advantage of all the preceding information, we decided to purify *E. coli* ATPase using conditions which may afford a maximal stability of the enzyme. The purification procedure should avoid any extreme change of pH, ionic strength and concentration of divalent cations. Molecular sieving appeared to be the most suitable method. Fig. 3a illustrates the Sephadex G-200 elution profile of protein and ATPase activity of a crude soluble ATPase. The peak of activity coincided with a shoulder at 278 nm (also present at 206 nm). Fig. 3b illustrates the Sepharose elution profile showing in this case a highly symmetrical peak of protein. Both preparations were stimulated by trypsin (see Table II)

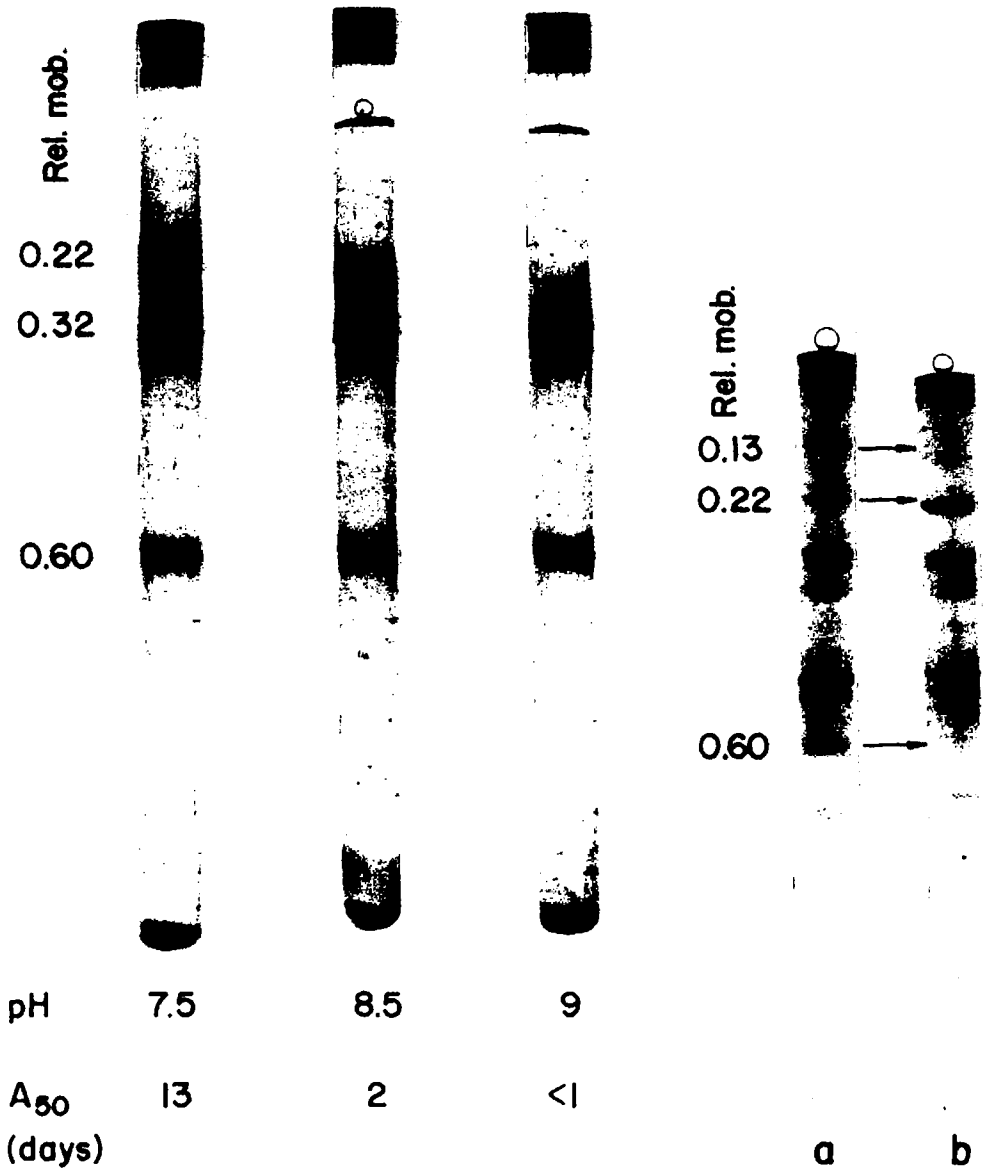

Fig. 1. ATPase staining of the crude enzyme from *E. coli* showing the influence of the pH value during storage at 4°C. The half-life of enzyme activity (A_{50}) is given in number of days under the respective pH value. Electrophoresis was run with about 50 μg protein in the Tris/glycine system (pH 8.5 ± 0.2) as described under Materials and Methods. Anode at bottom. For other details see that section and the rest of the text.

Fig. 2. Influence of Mg^{2+} on the stability of the protein pattern of crude *E. coli* ATPase. Samples (200—250 μg protein/ml, 30 mM Tris · HCl, pH 7.5) were stored at 4°C for 25 days with 3 mM $MgCl_2$ (gel b) or without the divalent cation (gel a). Electrophoresis was carried out as described in Fig. 1. Anode at bottom. For other details see the text.

to an extent similar as that of soluble crude ATPase [9]. Fig. 4 compares the protein patterns in the Tris/acetate (pH 7.5) gel system of the crude extract

TABLE I

STABILITY OF SOLUBLE ATPase FROM *ESCHERICHIA COLI* K 12 AS A FUNCTION OF Mg^{2+} CONCENTRATION

Crude ATPase (200—250 μg protein/ml) in 30 mM Tris · HCl (pH 7.5) with different concentrations of Mg^{2+} (values indicated in the table) was stored at 4°C and its residual activity at different times was measured as described in Materials and Methods. For other details see the text. The results are expressed as percentage of ATPase activity at each day of storage taken as 100 that of one freshly obtained preparation.

Mg^{2+} (mM)	Time (days)				
	4	8	12	18	24
0	78	60	50	35	24
0.5	90	86	76	52	52
1	90	87	80	62	55
3	91	92	82	78	73
5	100	100	100	96	95

with that of the Sephadex G-200 preparation. Note that this preparation was highly enriched in component a although component c was still present. The Sepharose step was important to obtain an homogeneous protein consisting only of component a (see Fig. 5).

Table II outlines the purification procedure. The increase in specific activity after each step was consistent with that one may expect from the protein purification revealed by gel electrophoresis analysis (compare Figs. 4 and 5). However, the increase in specific activity after the Sepharose step was not so high as one could expect from the yield of this step, suggesting that some inactivation had occurred. Further attempts to purify the protein (e.g. by subsequent gel chromatography, preparative gel electrophoresis) did not increase its specific activity but rather decreased it. The inactivation of the purified protein is being currently investigated.

Gel electrophoresis analysis of purified ATPase from E. coli K 12, strain 414

Fig. 5 illustrates the electrophoretic patterns at neutral pH and two acrylamide concentrations of the Sepharose preparation. The protein profiles which superimposed those of ATPase activity showed the homogeneity of this preparation. The splitting in two peaks (submicroheterogeneity) was reminiscent of that reported for *Micrococcus lysodeikticus* ATPase [20]. Like in that enzyme, the submicroheterogeneity of *E. coli* ATPase was masked, i.e. a single peak was obtained, when higher amounts of protein were used [20]. The analysis of pure *E. coli* ATPase by gel electrophoresis at pH 8.5 (Tris/glycine) induced the appearance of several components (see inset of Fig. 5).

The possibility that the ATPase profile may be influenced by the electrophoretic conditions themselves was tested by analyzing the ATPase under different pH and buffer system. The pH values were selected near to neutrality because of the unstabilizing effect of alkaline pH (see above). Each electrophoretic buffer was used at two different concentrations (see Materials and Methods) to detect possible buffer-protein interactions [21]. The NaH_2PO_4/Na_2HPO_4 system in the concentrations used by us (see Materials and Methods) had very bad buffering properties for electrophoresis as shown by the rapid pH

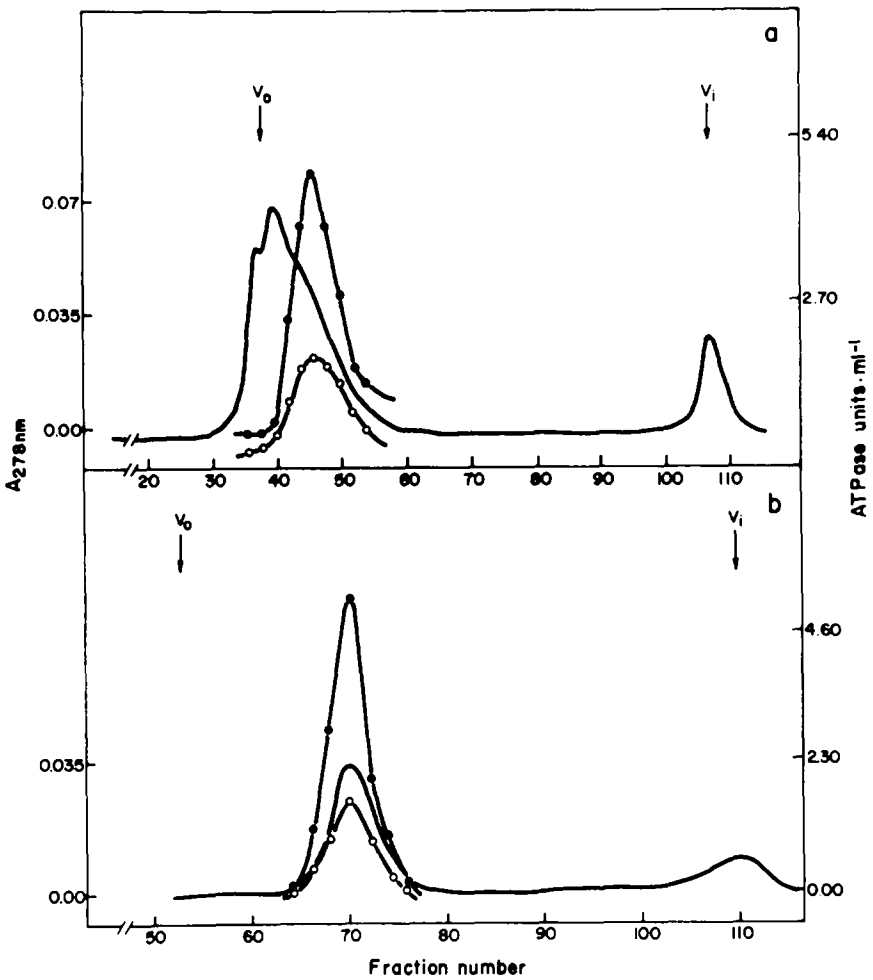

Fig. 3. Purification of *E. coli* ATPase by molecular sieving. Part a represents the protein and ATPase activity elution profiles of a Sephadex G-200 column (for characteristics see Materials and Methods). Crude soluble ATPase (80—90 mg protein) was charged into the column and eluted with 30 mM Tris · HCl (pH 7.5), 0.3 mM dithiothreitol, 3 mM $MgCl_2$ at a flow rate of 40 ml/h. Fractions of 12.8 ml were collected. The peak of ATPase activity (fraction 41—50) was pooled and concentrated by ultrafiltration with a Diaflo PM-10 membrane under N_2 at 1—1.5 kg/cm^2. Part b, protein and ATPase elution of the Sephadex G-200 ATPase peak (8—10 mg protein) on Sepharose 4B (for the characteristics of the column see Materials and Methods); the elution buffer was the same as above but at a flow rate of 15 ml/h and fractions of 4 ml were collected. The ATPase peak was processed as above to a final concentration of 1.5—2 mg protein/ml. Protein profiles (———) were obtained by continuous monitoring of the effluent with an Uvicord III LKB. For definition of ATPase units see Table II, o———o, basal ATPase; •———•, trypsin-stimulated ATPase.

changes occurring in the buffer chambers. Higher phosphate molarities were avoided because the effect of high ionic strength (see above). The other three systems, i.e. Tris/diethylbarbituric acid, Tris/acetate and HEPES/imidazole, had adequate buffering properties. The Tris/diethylbarbituric acid system had a strong poisoning effect on the enzyme as shown by a decrease in the proportion of the slowest moving ATPase band when the highest concentration of buffer was used (results not shown). The relative mobility of the main ATPase

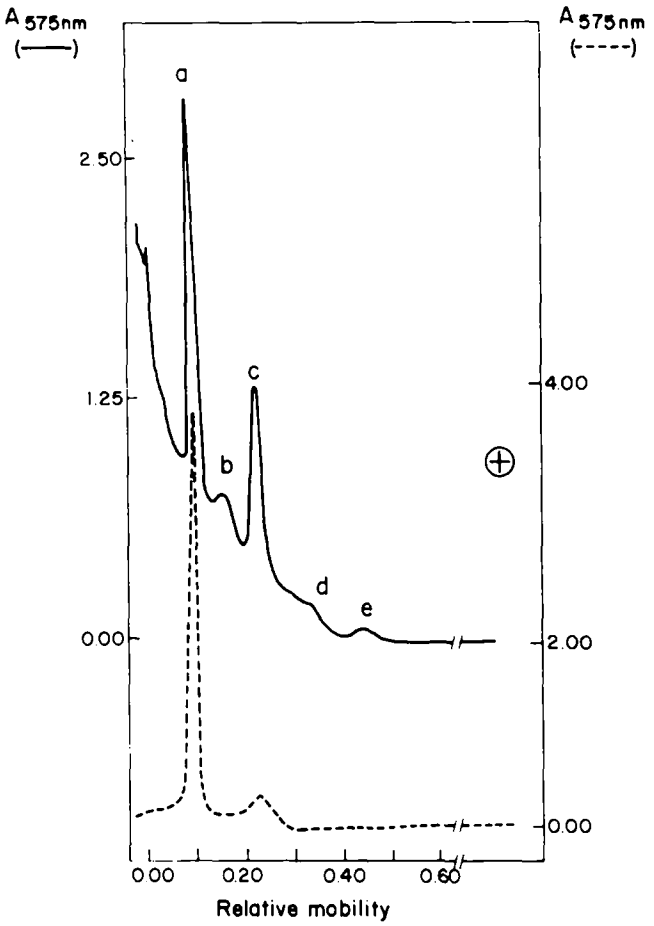

Fig. 4. Densitometric scannings of the protein patterns of crude (———) and Sephadex G-200 ATPase preparations (- - - - - -) from *E. coli* K 12 strain 414. About 50 μg protein were electrophoresed in 7% acrylamide gels using the Tris/acetate system (pH 7.5). For experimental details see Fig. 3 and Table II. Migration was from left to right (anode).

TABLE II

SCHEME OF PURIFICATION OF MEMBRANE ATPase FROM *E. COLI* K 12 STRAIN 414

For experimental details see Materials and Methods and Fig. 3.

Preparation	Protein (mg)	ATPase				
		Total units *		Specific activity **		Basal Stimulated
		+ Trypsin	— Trypsin	+ Trypsin	— Trypsin	
Membranes	480	576	288	1.2	0.6	0.5
Crude soluble	84	344	118	4.1	1.4	0.34
Sephadex G-200	7.5	281	102	37.5	13.6	0.36
Sepharose 4B	2.0	100	40	50.0	20.0	0.40

* Unit is defined as the amount of enzyme able to liberate 1 μmol P_i/min at 37° C.

** Specific activity = units/mg protein.

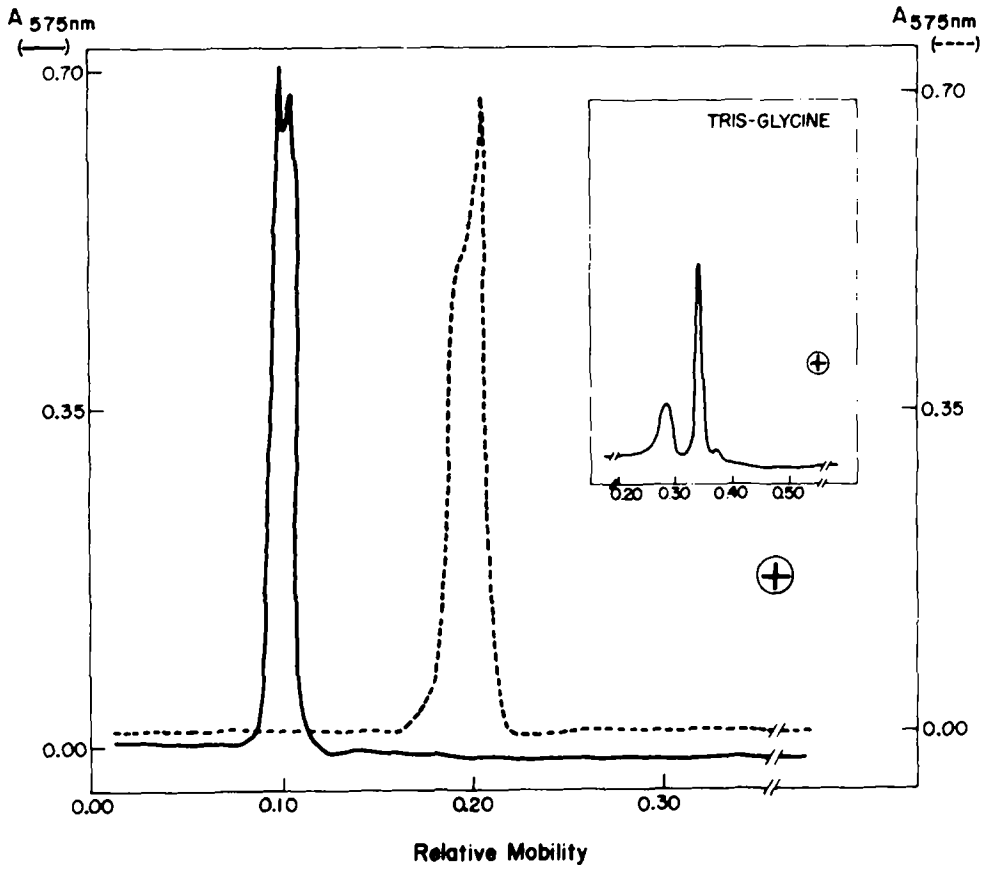

Fig. 5. Densitometric scannings of the protein patterns of the pure *E. coli* ATPase. About 15 μg protein were electrophoresed in the Tris/acetate system (pH 7.5), ———, 7% acrylamide, - - - - -, 5% acrylamide. The inset shows the pattern of the same protein analyzed in the Tris/glycine system (pH 8.5). Migration was in all cases from left to right (anode).

band as well as the number of protein bands differed depending on the systems used.

Fig. 6 illustrates the results with the ATPase band after isolation from the Tris/acetate system (semipreparative electrophoresis). When the re-purified band was subsequently analyzed in the same system at two acrylamide concentrations (gels a and b), 98% of the stain apparently corresponded to a single band. This result, therefore, confirmed that the homogeneity of the ATPase band in this system was a real fact. However, the Tris/glycine system splitted again the band into several components (gels c and d). On the other hand, the HEPES/imidazole system had a similar but less dramatic effect (gels e and f). A predominant band was still observed, although the presence of polydisperse material running behind the band suggested the importance of buffer-protein interactions in this system. Moreover, the presence of a fast-moving component supports the idea that these interactions may be, at least partially, quite strong.

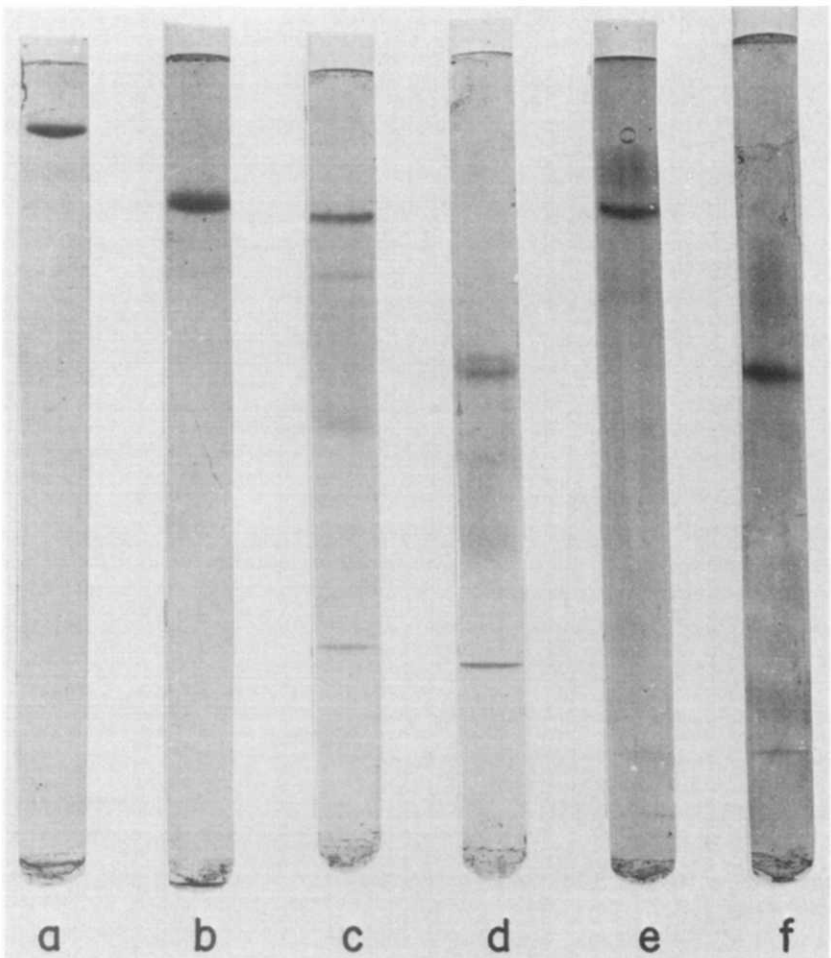

Fig. 6. Electrophoresis under different conditions of the main ATPase band purified by semipreparative electrophoresis at pH 7.5 in the Tris/acetate system. The purification procedure and different electrophoretic systems were detailed under Materials and Methods. Gel a, 7% acrylamide and gel b, 5% acrylamide, both run in Tris/acetate; gel c, 7% acrylamide and gel d, 5% acrylamide run in Tris/glycine; gel e, 7% acrylamide and gel f, 5% acrylamide, analyzed in the HEPES/imidazole system. Migration was towards the bottom.

Sodium dodecyl sulphate electrophoresis of purified ATPase from E. coli K 12 strain 414 and molecular weights of its subunits

The subunit pattern of the purified ATPase was examined by sodium dodecyl sulphate gel electrophoresis. In Tris/glycine (Fig. 7) we obtained five subunits grossly corresponding to those reported by Futai et al. [7] and Bragg et al. [22] plus a component x whose significance is unknown [20]. Small differences in the molecular weight of these subunits (see Table III) were found in our case with respect to previous work [7—9,22—24]. Another difference was the change in the relative proportion of the γ and δ subunits [8,24]. In dodecyl sulphate/Tris/acetate electrophoresis carried out at neutral pH, we only detected four subunits (Fig. 7). This result resembled that obtained by several groups

TABLE III

TYPE AND MOLECULAR WEIGHT OF *E. COLI* K 12 STRAIN 414 AND *M. LYSODEIKTICUS* ATPase SUBUNITS IN RELATION TO THE SODIUM DODECYL SULPHATE GEL ELECTROPHORESIS SYSTEM AND THEIR COMPARISON WITH ATPase SUBUNITS OF DIFFERENT *E. COLI* STRAINS

Experimental details in Materials and Methods and Fig. 7. The molecular weights in the Tris/glycine system are calculated in Student's *t* distribution and expressed as means ± confidence limits with a 95% probability from five different experiments.

Subunit	*E. coli* 414 ATPase		*M. lysodeikticus* ATPase *		*E. coli* 482 ATPase Bragg and Hou [5] (0.1 M phosphate, pH 7.2)	*E. coli* ML 308-225 ATPase Futai et al. [7] (phosphate, pH 7.0)
	Tris/acetate (pH 7.5)	Tris/glycine (pH 8.5)	Tris/acetate (pH 7.5)	Tris/glycine (pH 8.5) **		
x	112 000	120 000	—	—	—	—
α	66 000	54 000 ± 2000	56 000	52 500 ± 5000	56 500—57 000	58 000
β	60 000	48 000 ± 3000	50 000	47 500 ± 4500	50 800—52 700	52 000
γ	28 000	30 000 ± 2000	32 000	41 500 ± 2000	29 000—31 000	31 000
δ	—	21 000 ± 2000		28 500 ± 2000	18 900—23 000	20 000
ε	13 000	13 000			10 000—13 000	12 000

* Purified as indicated in ref. 14.

** Data from ref. 14. The γ subunit showed variations depending on the enzyme preparation and/or analytical conditions.

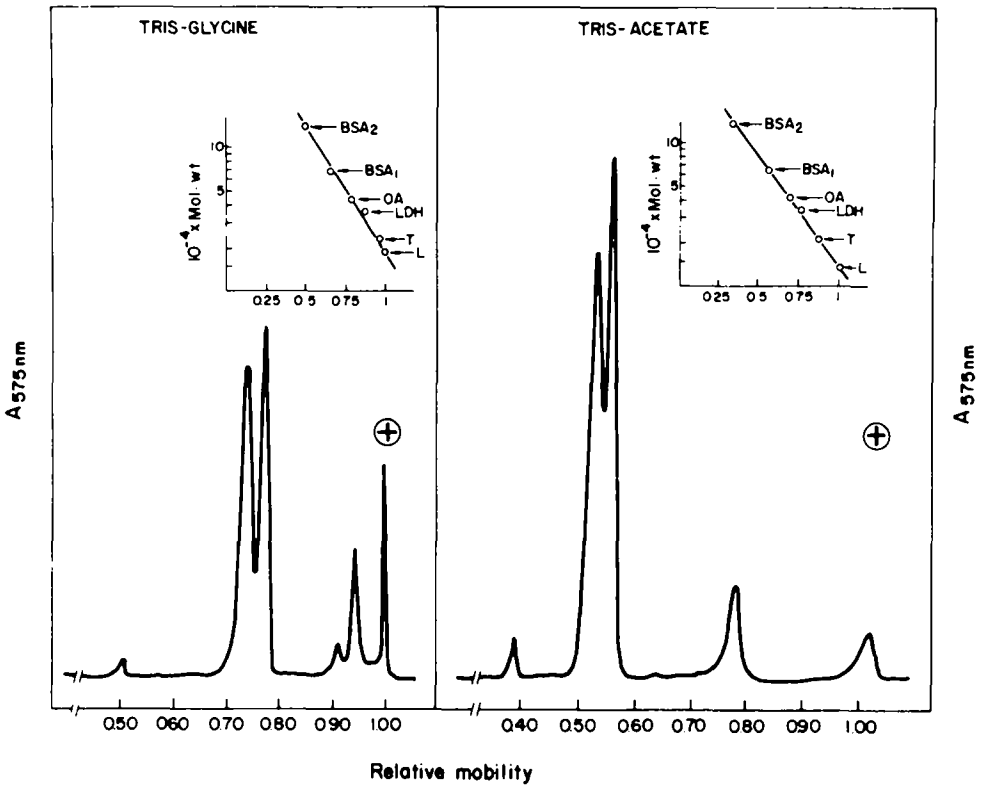

Fig. 7. Sodium dodecyl sulphate gel electrophoresis of pure *E. coli* ATPase in two buffer systems using 7% acrylamide gels. Samples (25 μg protein) were treated and electrophoresed as described under Materials and Methods. The insets represent the calibration curves obtained in both systems with proteins of known molecular weights. L, lysozyme; T, trypsin; LDH, lactate dehydrogenase; OA, ovoalbumin; BSA_1, bovine serum albumin monomer; BSA_2, bovine serum albumin dimer. For further details see the text.

[7—9,22,23]. However, the molecular weights of the major subunits were slightly higher than those reported by other authors [8,9,22—24] and ourselves working at alkaline pH (see Table III). The common component, x, and the γ subunit gave similar molecular weights in both systems (Table III). Moreover, the molecular weights of *M. lysodeikticus* ATPase subunits also gave similar values independently of the buffer system used (see Table III).

Discussion

The present work has provided some basis to understand the extreme instability of the ATPase of the *E. coli* K 12 strain 414 studied by us.

The fact that we have been able to increase considerably the half-life of this unstable ATPase may be of general interest and applicability. Nelson et al. [8] used ATP to protect the ATPase from *E. coli* K 12, strain 428. We have shown that another component of the substrate, i.e. Mg^{2+}, protected *E. coli* 414 ATPase. This protection presented evident economical advantages. Moreover, the prolonged interaction of ATPases with ATP may induce some ATP hydrolysis, i.e. there is an ATP hydrolase activity independent of added divalent cations in

ATPases (refs. 25 and 26 and Carreira, J. and Muñoz, E., unpublished results). The ATP hydrolysis may then influence the ATPase in a complex and yet unpredictable way [27,28]. In relation to the protective effect of Mg^{2+} on *E. coli* ATPase, it is worth to mention that the divalent cation appeared to interact reversibly with the enzyme and to bring on it marked molecular changes. This is interesting in connection with recent findings on the presence of Zn^{2+} as an integral part of the molecule of *E. coli* ATPase [29] and on the ability of divalent cations to induce conformational changes in energy-transducing proteins (refs. 24, 30—32, and Carreira, J. and Munoz, E., unpublished results). From these results, it is tempting to think that the sigmoid shape previously observed in kinetic studies [12] of the EDTA-soluble ATPase from this *E. coli* strain may be due to the effect of EDTA present in the preparation. As a matter of fact, after titrating the EDTA, the progress curve of ATPase activity changed its sigmoidal shape [12] to hyperbolic. Except for this difference, the purified enzyme resembled in its kinetic properties to the soluble enzyme previously studied by Carreira and Muñoz [12]. On the other hand, the specificity of the enzyme was similar to that reported by other authors for ATPases of different *E. coli* strains [3,6].

The analytical conditions of gel electrophoresis influenced the apparent homogeneity of the ATPase purified from *E. coli* K 12 strain 414. Microheterogeneity and existence of forms have recently been found to be an intrinsic property of ATPase coupling factor by two independent laboratories working with F_1 factors from chloroplasts and mitochondria [27] and two different bacteria [21,27,33]. The ATPase from *E. coli* 414 did not show a marked heterogeneity when analyzed by electrophoresis in Tris/acetate. Conversely, the heterogeneity induced in it by the Tris/glycine electrophoretic system was extrinsic and more drastic than that revealed by the above-mentioned coupling factors. A microheterogeneity of this *E. coli* ATPase reminiscent of that observed with *M. lysodeikticus* ATPase [21,33] was found sporadically from preparation to preparation and reflected itself, when analyzed in the Tris/glycine system, in the appearance of bands of relative mobility 0.19—0.24 (low specific activity, 7—15 μmol $P_i \cdot min^{-1} \cdot mg\ protein^{-1}$) and relative mobility 0.25—0.27 (high specific activity forms; 30—80 $\mu mol \cdot min^{-1} \cdot mg\ protein^{-1}$).

The molecular basis of the high instability of the ATPase from *E. coli* K 12 strain 414 is not yet clearly established. Most of the problem arose from strain characteristics (i.e. was due to intrinsic factors) because we needed the EDTA-alkaline pH extraction procedure to attain total ATPase solubilization [9], and this procedure proved to be deleterious for the enzyme. Moreover, the use of other solubilization procedures such as osmotic shock-depletion of Mg^{2+}, detergents (ref. 9 and unpublished observations) gave low percentages of ATPase activity of even higher instability (see also Introduction).

However, the purification procedure developed by us gave relatively satisfactory results in terms of yield and specific activity. On the basis of this last parameter, the purified ATPase from this *E. coli* K 12 strain was a little less active than that purified by Nelson et al. [8] and Futai et al. [7] but equally or more active than the preparations obtained by other workers [1,3,5,6]. The dodecyl sulphate subunit pattern of this *E. coli* ATPase also reflected the high degree of purity of the protein. However, it is worth noting that the subunit

pattern also showed differences comparing the Tris/acetate (pH 7.5) system with the alkaline Tris/glycine. In the first system, the δ subunit was absent and the molecular weights of α and β polypeptides were higher. The δ subunit in other *E. coli* ATPases was also present or absent in the purified enzymes but its presence did depend on the purification procedure and not on the electrophoretic conditions [5—8]. These small molecular differences might account for the different stability of *E. coli* ATPases from various origins. We do not yet know whether these molecular differences may reflect different structures determined genetically or be a consequence of alterations produced by protein manipulation. These possibilities should be explored in future research by studying intrinsically and extrinsically induced differences at the level of the supramolecular organization of ATPase systems and/or of their protein and non-protein components (nucleotides, sugars, lipids). The marked heterogeneity following total inactivation in the test tube of ATPase from *E. coli* 414 appears to be a peculiar property of this enzyme induced by mild extrinsic factors, i.e. moderately alkaline pH and/or ionic strength, presence of certain ions.

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References

1 Evans, D.J. (1970) J. Bacteriol. 104, 1203—1212
2 Roisin, M.P. and Kepes, A. (1973) Biochim. Biophys. Acta 305, 249—259
3 Kobayashi, H. and Anraku, Y. (1972) J. Biochem. Tokyo 71, 387—399
4 Davies, P.L. and Bragg, P.D. (1972) Biochim. Biophys. Acta 266, 273—284
5 Bragg, P.D. and Hou, C. (1972) FEBS Lett. 28, 309—312
6 Hanson, R.L. and Kennedy, E.P. (1973) J. Bacteriol. 114, 772—781
7 Futai, M., Sternweis, P.C. and Heppel, L.A. (1974) Proc. Natl. Acad. Sci. U.S. 71, 2725—2729
8 Nelson, N., Kanner, B.I. and Gutnick, D.L. (1974) Proc. Natl. Acad. Sci. U.S. 71, 2720—2724
9 Carreira, J., Leal, J.A., Rojas, M. and Muñoz, E. (1973) Biochim. Biophys. Acta 307, 541—556
10 De Corao, M., Serrano, J.A., Leal, J.A., Puig, J. and Munoz, E. (1974) Microbiol. Esp. 27, 283—298
11 Vambutas, V.K. and Racker, E. (1965) J. Biol. Chem. 240, 2660—2667
12 Carreira, J. and Muñoz, E. (1975) Mol. Cell. Biochem. 9, 85—95
13 Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265—275
14 Andreu, J.M., Albendea, J.A. and Muñoz, E. (1973) Eur. J. Biochem. 37, 505—515
15 Muñoz, E., Larraga, V., Azocar, O. and Carreira, J. (1975) in Biomembranes Lipids, Proteins and Receptors (Burton, R.M. and Packer, L., eds.), Chapter 13, pp. 232—252, Bi-Science Publ. Divison, Webster Groves, Missouri
16 Williams, D.E. and Reisfeld, R.A. (1971) Ann. N.Y. Acad. Sci. 121, 373—381
17 Fairbanks, G., Steck, T.L. and Wallach, D.F. (1971) Biochemistry 10, 2606—2617
18 Dietzel, W., Kopperschlager, G. and Hoffman, E. (1972) Anal. Biochem. 48, 617—620
19 Weinbaum, G. and Markman, R. (1966) Biochim. Biophys. Acta 124, 207—209
20 Carreira, J., Andreu, J.M., Nieto, M. and Muñoz, E. (1976) Mol. Cell. Biochem. 10, 67—76
21 Cann, J. and Good, W.B. (1968) in Advances in Enzymology (Nord, F.F., ed.), Vol. 30, pp. 139—177, Interscience, New York
22 Bragg, P.D., Davies, P.L. and Hou, C. (1973) Arch. Biochem. Biophys. 159, 664—670
23 Kobayashi, H. and Anraku, Y. (1974) J. Biochem. Tokyo 76, 1175—1182
24 Vogel, G. and Steinhart, R. (1976) Biochemistry 15, 208—216
25 Adolfsen, R. and Moudrianakis, E.N. (1973) Biochemistry 12, 2926—2933

26 Muñoz, E., Salton, M.R.J., Ng, M.H. and Schor, M.T. (1969) Eur. J. Biochem. 7, 490—501
27 Adolfsen, R., McClung, J.A. and Moudrianakis, E.N. (1975) Biochemistry 14, 1727—1735
28 Ayala, J., Carreira, J., Nieto, M. and Munoz, E. (1976) Mol. Cell. Biochem., in the press
29 Sun, I.L. and Crane, F.L. (1975) Biochem. Biophys. Res. Commun. 65, 1334—1342
30 Bakker-Grunwald, T. (1974) Biochim. Biophys. Acta 347, 141—143
31 Moyle, J. and Mitchell, P. (1975) FEBS Lett. 56, 55—61
32 Sone, N., Yoshida, M., Hirata, H. and Kagawa, Y. (1975) J. Biol. Chem. 250, 7917—7923
33 Nieto, M., Muñoz, E., Carreira, J. and Andreu, J.M. (1975) Biochim. Biophys. Acta 413, 394—414