EFFECTS OF PROTEOLYTIC FRAGMENTATIONS ON THE ACTIVITY OF THE MITOCHONDRIAL NATURAL ATPase INHIBITOR

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1. Introduction

The so-called natural ATPase inhibitor, IF_1, is a peptide of low M_r (10 000 [1,2]), which has been purified from mitochondria of a number of species [3–8]. Under appropriate conditions (slightly acidic pH, Mg-ATP), IF_1 binds to F_1, the hydrophilic part of the ATPase complex, and blocks its hydrolytic activity [1,3,9,10]. As typically illustrated for beef heart F_1-ATPase, the inhibitory activity of IF_1 is associated with its specific binding to the β subunit of F_1 [11,12]. Further, although each mole of F_1 contains 2 or possibly 3 β subunits, the binding of 1 IF_1 to only 1 β subunit is sufficient to promote full inhibition of the hydrolytic activity of F_1 [11]. Other structural aspects concerning especially the interaction of IF_1 with the β subunit of F_1 are virtually lacking. In [13] tryptic fragmentation of IF_1 close to the N-terminus yielded a peptide of 8000 M_r, deprived of the N-terminal amino acid sequence, which is still capable of interacting with F_1 and bringing about inhibition of the hydrolytic activity. This peptide referred to as T_1 contains the same C-terminus as IF_1.

The detailed effects of a limited tryptic cleavage of IF_1 are reported here. Beside the active 8000 M_r peptide (T_1) that is the first cleavage product of IF_1 to accumulate upon incubation with trypsin, we detected a closely related fragment of 7500 M_r (T_2) endowed with inhibitory activity, and to demonstrate that the 7500 M_r peptide is issued from a two-step proteolytic process involving first the accumulation of the transient 8000 M_r fragment and its subsequent conversion into the 7500 M_r peptide. The peptide bond cleavages yielding fragments T_1 and T_2 were identified, and the amino acid sequence around these sites of cleavage reported here. Among other proteolytic enzymes that were investigated, clostripain and thrombin were found to generate from IF_1, a peptide of 8000 M_r, active as ATPase inhibitor and probably identical to T_1. Chymotrypsin and *Staphylococcus aureus* V8 protease which attack IF_1 to remove larger peptide fragments than trypsin, clostripain or thrombin yielded inactive peptides. Removal of amino acids from the C-terminus of IF_1 by carboxypeptidase P resulted in some loss of enzymatic activity. In summary, the entire molecule of IF_1 is not necessary for functioning as ATPase inhibitor. In fact, a substantial sequence of amino acids corresponding to 2500 M_r can be removed from the N-terminus of IF_1 without loss of inhibitory activity; the other regions of the molecule are more critical for activity.

Abbreviation: TPCK-trypsin, trypsin-treated by L-(1-tosylamido-2-phenylethyl-chloromethylketone)

2. Materials and methods

Acrylamide, bis-acrylamide and TMED were purchased from Eastman Kodak, SDS from Serva, *Staphylococcus aureus* V8 protease and α-chymotrypsin from Miles, clostripain from Precibio, TPCK-trypsin from Worthington Biochemicals, the soybean trypsin inhibitor, the Kunitz pancreas inhibitor from Sigma, thrombin from Institut de Sérothérapie Hémopoïétique, carboxypeptidase Y from Pierce, and carboxypeptidase P from Takara-Shuzo, Kyoto.

Beef heart mitochondria and AS particles (submitochondrial particles depleted of natural inhibitor)

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were prepared as in [14] and [15] respectively. Beef heart ATPase inhibitor (IF_1) was purified by the method in [3], as modified [16] for the ethanol fractionation step.

The inhibitory activity of IF_1 and IF_1 fragments on the ATPase activity of AS particles was assayed as follows: AS particles were incubated with IF_1 in presence of 1 mM Mg-ATP at 30°C (pH 6.8) for 15 min to allow binding of IF_1 to the particles. The residual ATPase activity in AS particles after IF_1 binding was measured upon addition of the ATP regenerating medium containing 0.1 M Tris–SO_4 pH 8.0, 20 mM ATP, 10 mM $MgCl_2$, 20 mM phosphoenolpyruvate, 30 μg pyruvate kinase, for 10 min at 30°C. The reaction was stopped by perchloric acid, and the amount of P_i released was determined [17]. Protein concentration was determined either by the biuret method [18] or the Bradford technique with Coomassie blue G-250 [19]. Bovine serum albumin was used as a standard.

Polyacrylamide–SDS slab gels were made as in [20], using a 10% stacking gel and a 20% separating gel. Electrophoresis was run at pH 8.9 for 16 h at a 125 V constant voltage. M_r markers were: muscle triose phosphate isomerase (27 000) from Boehringer; soybean trypsin inhibitor (20 500) from Sigma; horse heart cytochrome *c* (12 400) from Sigma; horse heart myoglobin (17 000) and its CNBr cleavage products (14 000, 8100, 6200, 2500) from BDH; bovine lung aprotinin (6500) from Boehringer; β chain of insulin (3400) from Serva. Isofocusing on slab gel was run in a LKB Multiphor Unit, as in [21]. The gel contained 5% acrylamide, 8 M urea and 2% ampholines (pH 3.5–10) (from LKB). Proteins were stained by Coomassie blue R-250.

Thrombin attack of IF_1 was performed at 37°C in 0.2 M $NH_4CH_3CO_2$ (pH 7.9) at a thrombin/IF_1 ratio of either 1/150 for 30 min, as used for fibrinogen clotting, or 1/50 for 24 h incubation. *Staphylococcus aureus* V8 protease digestion of IF_1 was carried out at 30°C in 30 mM NH_4HCO_3 (pH 7.9) using a protease/IF_1 ratio of 1/50. Carboxypeptidase Y and carboxypeptidase P were used at 37°C with a protease/IF_1 ratio of 1/50 in 0.1 N pyridine/acetate buffer (pH 5.5) and in 0.1 N pyridine/formate buffer (pH 2.5), respectively. α-Clostripain attack of IF_1 required activation of clostripain by reduction of the disulfur bridges between its 2 subunits with dithiothreitol [22]. A clostripain/IF_1 ratio of 1/200 at 37°C in 20 mM NH_4HCO_3 (pH 7.9) was used.

Partial hydrolysis of IF_1 by TPCK-trypsin was followed at 10°C, in 50 mM $NH_4CH_3CO_2$ and 1% NH_4HCO_3 (pH 7.8) using a trypsin/IF_1 ratio of 1/100. The proteolytic activity was stopped by addition of a 3-fold excess of soybean tryptic inhibitor with respect to trypsin. Limited hydrolysis of IF_1 by α-chymotrypsin was done at 20°C, using a chymotrypsin/IF_1 ratio of 1/200, and the same medium as for trypsin incubation. The reaction was terminated either by addition of a 5–10-fold excess of the Kunitz pancreas inhibitor, or by heating at 100°C for 5 min, followed by lyophilisation for samples subjected to SDS gel electrophoresis. The kinetics of action of the preceeding enzymes was studied by withdrawing aliquots of the incubation medium as a function of time. In the absence of specific inhibitors, the proteolytic activities were blocked by heating for 5 min at 100°C. After heating, IF_1 retained all its biological properties. The hydrolytic products were characterized by their M_r, and by their inhibitory capacity with respect to the ATPase activity.

The methods used for amino acid sequence were described in [13]. Correct alignment of the tryptic peptides in the N-terminal region of the IF_1 molecule was made possible by digestion of IF_1 with the *S. aureus* V8 protease.

3. Results and discussion

3.1. *Characterisation of beef heart IF_1*

The purity of beef heart IF_1 was assessed by electrophoresis on 20% acrylamide gel with SDS. IF_1 migrated as a single band, with app. $M_r \sim 10\,000$, as estimated by comparison with comigrating standard markers. The presence of 7 M urea brought no change in this behaviour. A pH_i value between 7.3 and 7.5 was determined for IF_1 by isofocusing in presence of ampholines on 5% acrylamide slab gel (section 2), agreeing well with [4].

3.2. *Inhibitory activity of proteolytic fragments derived from IF_1*

Staphylococcus aureus V8 protease is specific for cleavage of peptide bonds at the level of glutamyl residues. Incubation of *S. aureus* V8 protease with IF_1 at a ratio of 1/50 for 1 min at 30°C inactivated 50% of IF_1, and resulted in the degradation of half of IF_1 into a 5500 M_r inactive peptide (P_1) as shown by SDS gel electrophoresis. The other part of the IF_1

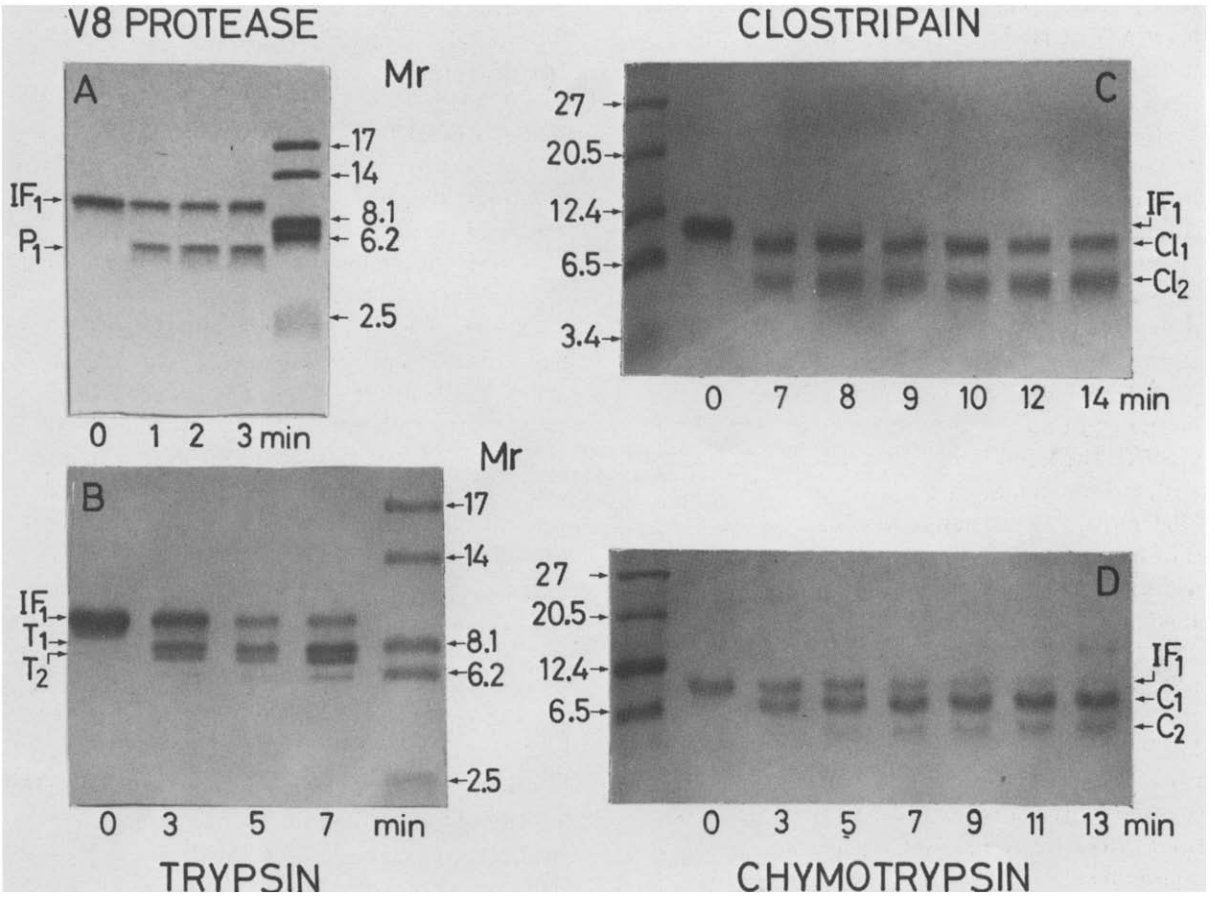

Fig.1. SDS gel electrophoresis of products obtained by digestion of beef heart IF_1 with proteolytic enzymes. Incubation media are described in section 2. (A) *Staphylococcus aureus* V8 protease; hydrolysis times, 0,1,2,3 min at 30°C (V8/IF_1, 1/50); (B) trypsin hydrolysis times, 0,3,5,7 min at 10°C (trypsin/IF_1, 1/100); (C) α-clostripain hydrolysis times, 0,7,8,9,10,12,14 min at 37°C (clostripain/IF_1, 1/200); (D) α-chymotrypsin hydrolysis times, 0,3,5,7,9,11,13 min at 20°C (chymotrypsin/IF_1, 1/200). The M_r values ($\times 10^{-3}$) of comigrating standard proteins are indicated (section 2).

molecule ($M_r \sim 4500$) was further digested into small size peptides as they were not visualized on gel (fig.1A).

When thrombin was used under the same conditions as those described for cleavage of fibrinogen into fibrin (section 2), no modification in the M_r and the activity of IF_1 could be detected. This suggested that the sequences specific for the action of thrombin were either absent, or not readily available in IF_1. However, when incubation was done for long periods (24 h at 37°C, at a thrombin/IF_1 ratio of 1/50), 50% of IF_1 was degraded into a 8000 M_r peptide which was still active (not shown).

Digestion of IF_1 by carboxypeptidase Y was very slow. Carboxypeptidase P was significantly more active. Incubation of IF_1 with carboxypeptidase P for 11 h at 37°C resulted in the release of a dozen of amino acids (1000 M_r), as revealed by SDS gel electrophoresis, and concomitantly the inhibitory activity of the digest was decreased by 50%, indicating that the C-terminal amino acid sequence plays a critical role in the inhibitory activity of IF_1. This conclusion must be qualified, however, because of the thermic treatment used to stop the action of carboxypeptidase on the C-terminus of IF_1, and the possibility that the carboxypeptidase fragment of IF_1 could be more thermolabile than IF_1. In fact the C terminal end of IF_1 is rich in acid and basic amino acid residues ([23], unpublished) that could contribute to thermal stability of IF_1 by forming salt bridges; examples of heat stability in thermophile enzymes,

Table 1
Size and enzymatic activity of the main products obtained by partial proteolysis of IF_1 with trypsin, α-clostripain and chymotrypsin

Enzyme	Incubation time	Products	M_r (approx.)	Recovery (%)	Inhibitory activity
TPCK-trypsin	7 min	T_1	8000	≃ 80	+
	15 min	T_2	7500	≃ 50	+
α-clostripain	1 min	Cl_1	8000	50	+
	8 min	Cl_2	5500	50	0
α-chymotrypsin	3 min	C_1	7000	50	0
	10 min	C_2	5500	40	0

Experimental conditions for partial proteolysis of IF_1 are given in section 2. M_r-Values were estimated after SDS–polyacrylamide gel electrophoresis by comparison with comigrating standard M_r markers; note that they are approximate values. The recovery (in %) was estimated from Coomassie blue staining at 660 nm

due to salt bridges were discussed in [24].

Under the incubation conditions of temperature, enzyme/IF_1 ratio and time described for the above enzymes, α-chymotrypsin, TPCK-trypsin were found to quickly digest beef heart IF_1, resulting in small inactive peptides. It was possible, however, to slow down the initial kinetics of the digestion by increasing the ionic strength of the medium, decreasing the protease/IF_1 ratio, and lowering the temperature of the medium. Under well-defined conditions, large size fragments of IF_1 were recovered, some still being able to inhibit ATPase activity. This was the case for large IF_1 fragments obtained by partial trypsin and clostripain proteolysis (table 1). The recovery was never 100%, due to the fact that a fraction of the large peptides produced during hydrolysis of IF_1 was further degraded.

As shown in fig.1B, limited tryptic attack of IF_1, using a trypsin/IF_1 ratio of 1/100 at 10°C, resulted into the release of a 8000 M_r peptide, T_1, which was further cleaved to give a shorter T_2 product of M_r 7500. The inhibitory activity of the cleavage products of IF_1 on the ATPase of AS particles was assessed during the course of the digestion of IF_1 by trypsin. We routinely checked the relationship between the amount of the IF_1 digest used (containing T_1 and T_2) and the extent of inhibition of ATPase (fig.2). T_1 and T_2 were further identified as active fragments as follows. The tryptic incubation medium containing the mixture of non-digested IF_1 plus T_1, T_2 and smaller peptides was subjected to SDS–polyacrylamide gel electrophoresis. After separation of the peptides, the unstained gel was cut into 1 mm slices, which were soaked into 0.5 ml of a medium made of 0.25 M sucrose, 5 mM DTT, 4 mg BSA/ml for 16 h at 4°C to extract the products. Then 10 mM MOPS (pH 6.5), 0.5 mM ATP and 0.5 mM $MgCl_2$ were added to the extract with 25 μg AS particles at 30°C, to allow binding of IF_1 to F_1-ATPase, and the residual ATPase activity was measured with the ATP regenerating medium in section 2, at 30°C for 10 min. A control consisting of non-digested IF_1 was processed under the same conditions as above. Duplicate gels were stained by Coomassie blue. T_1 and very probably T_2 inhibit ATPase (as does IF_1) (fig.3). Therefore the entire molecule of IF_1 is not necessary for inhibitory activity. The 8000 M_r active peptide T_1 has the same

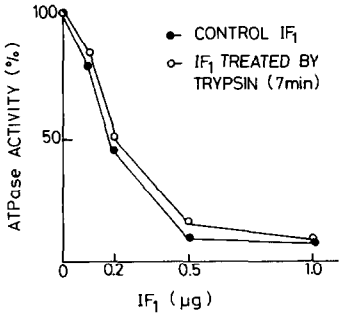

Fig.2. Effect of increasing concentrations of the tryptic cleavage products of IF_1 on the inhibition of ATPase activity of AS particles. The experimental conditions are given in section 2. IF_1 was subjected to trypsin hydrolysis as in fig.1B for 7 min, yielding a large percentage of T_1 and T_2 fragments.

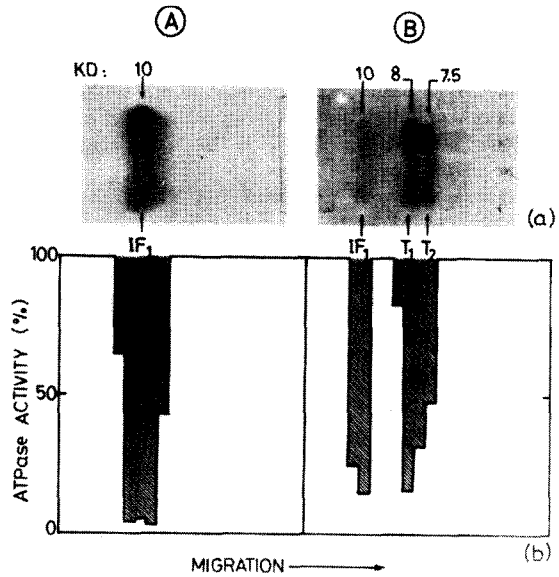

Fig.3. SDS gel electrophoresis of IF_1 (A) and products of partial digestion by trypsin (T_1, T_2) (B) See text for experimental conditions: (a) Coomassie blue staining; (b) inhibitory efficiency of the corresponding eluted bands. The inhibition is proportional to the size of the hatched bars.

C-terminal sequence as IF_1. Further, amino acid sequence analysis close to the N-terminal portion of IF_1, using small tryptic peptides and larger peptides obtained by the *S. aureus* V8 protease (submitted) indicated that T_2 was issued from T_1 by attack of the N-terminus of T_1. Therefore T_1 and T_2 are fragments of IF_1 essentially deprived of the N-terminal amino acid sequence and possessing an intact C-terminus.

As with trypsin, incubation of IF_1 with clostripain at 1/200 at 37°C yielded in 1 min incubation an active 8000 M_r (Cl_1) peptide, which was further cleaved into inactive shorter fragments (fig.1C). Trypsin, clostripain and thrombin probably release the same active 8000 M_r fragment by cleavage of the N-terminus of IF_1.

In contrast to the early products of digestion of IF_1 by trypsin, clostripain and thrombin, which were found to be active on F_1-ATPase, the earliest proteolytic derivative of IF_1 obtained by action of chymotrypsin (C_1) was inactive. Its M_r was 7000, corresponding to only a dozen amino acids less than the active T_1 [13]. C_1 was quickly degraded into a peptide of $M_r \sim 5500$, C_2 (fig.1D). Interestingly, *N*-bromosuccinimide, used as in [25], with 100-fold molar excess of the reagent over the tyrosine content of IF_1 for 4 h at 56°C in 80% formic acid, was also able to release a 5500 M_r peptide. Since IF_1 contains no tryptophan, and only 1 tyrosyl residue [13], this implied that cleavage of IF_1 by *N*-bromosuccinimide occurred at the level of that tyrosyl residue, virtually in the middle of the IF_1 molecule. As C_2 had the same M_r as the *N*-bromosuccinimide product of IF_1, cleavage by α-chymotrypsin to yield C_2 occurred at the level of the tyrosyl residue. This was confirmed

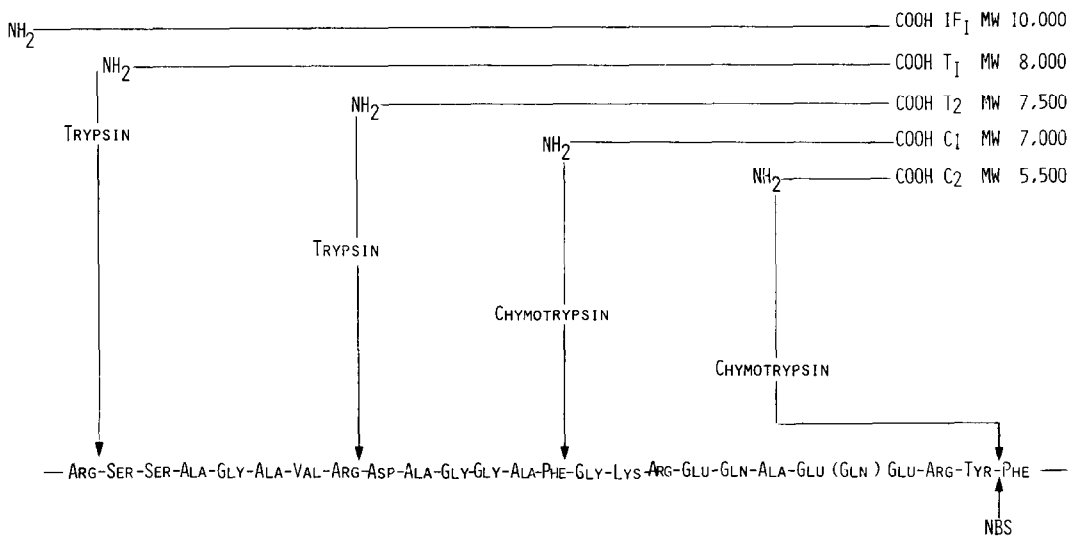

Fig.4. Sites of cleavage of IF_1 by trypsin, clostripain, thrombin and chymotrypsin in the N-terminal region of IF_1. Note that *N*-bromosuccinimide (NBS) cleaves the same bond of IF_1 (Tyr–Phe) as chymotrypsin, in the middle region of IF_1 (section 3).

by isolation of C_2, and identification of phenylalanine as N-terminal residue of C_2.

3.3. *Bond cleavages yielding T_1, T_2, C_1 and C_2*

We reported a short sequence of amino acids in IF_1 corresponding to a tryptic cleavage product in [13]; shortly thereafter, the full sequence of IF_1 was published [23]. The use of *S. aureus* V8 protease allowed us to solve the problem of alignment of short peptides obtained by treatment of IF_1 by trypsin and chymotrypsin. We essentially agree with [23] for the portion of the sequence given in fig.4, which illustrates the enzymatic cleavage sites in the N-terminal region of IF_1 by trypsin (yielding active T_1 and T_2) and by chymotrypsin (yielding inactive C_1 and C_2).

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References

[1] Pullman, M. E. and Monroy, G. (1963) J. Biol. Chem. 238, 3762–3769.
[2] Brooks, J. C. and Senior, A. E. (1971) Arch. Biochem. Biophys. 147, 467–470.
[3] Horstman, L. L. and Racker, E. (1970) J. Biol. Chem. 245, 1336–1344.
[4] Satre, M., de Jerphanion, M. B., Huet, J. and Vignais, P. V. (1975) Biochim. Biophys. Acta 387, 241–255.
[5] Landry, Y. and Goffeau, A. (1975) Biochim. Biophys. Acta 376, 470–484.
[6] Chan, S. H. P. and Barbour, R. L. (1976) Biochim. Biophys. Acta 430, 426–433.
[7] Ebner, E. and Maier, K. L. (1977) J. Biol. Chem. 252, 671–676.
[8] Cintron, N. M. and Pedersen, P. L. (1979) J. Biol. Chem. 254, 3439–3443.
[9] Klein, G., Satre, M. and Vignais, P. V. (1977) FEBS Lett. 84, 129–134.
[10] Gomez-Fernandez, J. C. and Haris, D. A. (1978) Biochem. J. 176, 967–975.
[11] Klein, G., Satre, M., Dianoux, A. C. and Vignais, P. V. (1980) Biochemistry 19, 2919–2925.
[12] Klein, G., Satre, M., Dianoux, A.-C. and Vignais, P. V. (1981) Biochemistry 20, 1339–1344.
[13] Dianoux, A.-C., Vignais, P. V. and Tsugita, A. (1981) FEBS Lett. 130, 119–123.
[14] Smith, A. L. (1967) Methods Enzymol. 10, 81–86.
[15] Racker, E. and Horstman, L. L. (1967) J. Biol. Chem. 242, 2547–2551.
[16] Kagawa, Y. (1974) Methods Membr. Biol. 1, 240–241.
[17] Fiske, C. H. and Subbarow, Y. (1925) J. Biol. Chem. 66, 375–400.
[18] Gornall, A. G., Bardawill, C. J. and David, M. M. (1949) J. Biol. Chem. 177, 751–766.
[19] Bradford, M. M. (1976) Anal. Biochem. 72, 248–254.
[20] Cabral, F. and Schatz, G. (1979) Methods Enzymol. 56, 602–613.
[21] Iborra, F. and Buhler, J. M. (1976) Anal. Biochem. 74, 503–511.
[22] Gilles, A. M., Imhoff, J. M. and Keil, B. (1979) J. Biol. Chem. 254, 1462–1468.
[23] Frangione, B., Rossenwasser, E., Penefsky, H. S. and Pullman, M. (1981) Proc. Natl. Acad. Sci. USA 78, 7403–7407.
[24] Perutz, M. F. (1978) Science 201, 1187–1191.
[25] Wachter, E. and Werhahn, R. (1979) Anal. Biochem. 97, 56–64.