Biochimica et Biophysica Acta, 593 (1980) 11—16

BBA 47939

FUNCTIONAL ARGININE RESIDUES AND CARBOXYL GROUPS IN THE ADENOSINE TRIPHOSPHATASE OF THE THERMOPHILIC BACTERIUM PS-3

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(Received February 19th, 1980)

Key words: ATPase; Arginine residue; Phenylglyoxal; Chemical modification; Adenine nucleotide binding; (Thermophilic bacterium PS-3)

Summary

Treatment of purified ATPase of the thermophilic bacterium PS-3 with the arginine reagent phenylglyoxal or with Woodward's reagent K, gave complete inactivation of the enzyme. The inactivation rates followed apparent first-order kinetics. The apparent order of reaction with respect to inhibitor concentrations gave values near to 1 with both reagents, suggesting that inactivation was a consequence of modifying one arginine or carboxyl group per active site.

ADP and ATP strongly protected the thermophilic ATPase against both reagents. GDP and IDP protected less, whilst CTP did not protect.

Experiments in which the incorporation of [^{14}C]phenylglyoxal into the enzyme was measured show that extrapolation of incorporation to 100% inactivation of the enzyme gives 8—9 mol [^{14}C]phenylglyoxal per mol ATPase, whilst ADP or ATP prevent modification of about one arginine per mol.

Introduction

The ATPase from the thermophilic bacterium PS-3 was recently purified and crystallized [1,2]. It is a remarkably stable enzyme that can be dissociated into five subunits and reconstituted from them [1,3,4]. This property has allowed

Abbreviations: Mes, 2-(*N*-morpholino)ethanesulfonic acid; TF_1, ATPase from the thermophilic bacterium PS-3.

study of the functions of each subunit. This ATPase (TF_1) is rather similar to the ATPases from mitochondria, chloroplasts and other bacteria [5] although not identical. For instance F_1 and CF_1 have eight sulfhydryl groups per mol and two and three disulfide bonds, respectively [6,7], whilst TF_1 has only three sulfhydryl groups, one in each α subunit [8] and no disulfide bond. Moreover, the sulfhydryl groups of CF_1 may play a role in the enzyme [7].

Chemical modification by dicarbonyl compounds of more than 100 enzymes has shown that they contain functional arginine residues (for a review see Ref. 9). It has been shown, for instance, that the ATPases from mitochondria [10] chloroplasts [11] and *Rhodospirillum rubrum* [12] have an essential arginine residue. In fact, an arginine residue in the β-subunit of F_1 was found in the vicinity of a tyrosyl group that covalently bound ATP-analog [13]. Moreover, X-ray crystallography of adenylate kinase clearly revealed several arginine residues surrounding the triphosphate group of ATP bound to the catalytic site [14]. The sensitivity of the forward and backward reactions of ATP synthesis in chloroplasts and chromatophores to dicarbonyl compounds is different and suggests the involvement of different arginine residues [12,15].

Chemical modification by Woodward's reagent K of several enzymes such as yeast phosphoglycerate kinase [16] has revealed the presence of essential carboxyl groups. The kinase was protected by MgATP. Modification of chloroplast coupling factor 1 by Woodward's reagent K inhibited its ATPase and coupling activity and suggested the involvement of carboxyl groups [17]. The soluble ATPases of a *Chromatium* strain D bacterium [18] and of mitochondria [19,20] were inhibited by dicyclohexylcarbodiimide and other carboxyl reagents.

The amino acid composition of TF_1 is different from that of other F_1's [8], so we thought it important to investigate its chemical modification to see if similar essential residues are present in TF_1.

The results reported in this paper suggest the presence of arginine residues and carboxyl groups in the active site of TF_1.

Materials and Methods

The growth conditions of the thermophilic bacteria PS-3 and the preparation of TF_1 were as described [1].

ATPase activity of TF_1 was determined at 60°C in a reaction medium (1 ml) containing 100 mM sodium borate buffer (pH 8.6), 5 mM $MgCl_2$ and 5 mM ATP. After 5 min preincubation, the reaction was started by adding TF_1 (1—2 μg) and stopped after 10 min incubation by adding trichloroacetic acid (5%). The phosphate liberated was determined colorimetrically according to Summer [21]. Protein was determined according to Lowry et al. [22].

Chemical modification of 0.1 mg/ml TF_1 by phenylglyoxal was carried out at 25°C in 100 mM borate buffer (pH 8) unless otherwise stated. Modification was stopped by dilution of TF_1 into the reaction medium. Chemical modification of TF_1 by Woodward's reagent K was carried out similarly, except that the buffer was Tricine/NaOH (pH 8) or Mes/NaOH (pH 6).

In the experiments with [^{14}C]phenylglyoxal, (3 mg/ml) TF_1 was incubated with 5 mM [^{14}C]phenylglyoxal in 100 mM *N*-ethyl morpholine/acetate buffer

(pH 8) at 25°C. Modification of samples taken at different times was stopped by centrifuging through Sephadex-G50 columns [23] equilibrated with the *N*-ethyl morpholine/acetate buffer. Radioactivity was counted in 5 ml scintillation fluid (5 g/l 2,3-diphenyloxazole, 100 g/l naphthalene in dioxan) in a Beckman LS-233 liquid scintillation spectrometer.

Nucleotides, phenylglyoxal and Woodward's reagent K (*N*-ethyl-5-phenylisoxazolium-3'-sulfonate) were obtained from Sigma Co.; [^{14}C]phenylglyoxal (26.2 mCi/mol) from Comisión Nacional de Energía Atómica (Argentina). All other reagents were of analytical grade quality.

Results and Discussion

Fig. 1 shows that incubation of TF_1 in borate buffer with several phenylglyoxal concentrations inhibited its ATPase activity. Phenylglyoxal is a reagent for specific chemical modification of arginine residues in proteins [9,24,25]. The derivative contains two phenylglyoxal moieties per guanidino group except in the presence of borate buffer where the ratio is probably 1 : 1 [26]. The inactivation followed pseudo-first-order kinetics and the reaction order with respect to phenylglyoxal concentration was 1.05 (inset Fig. 1), suggesting that inhibition was the consequence of 1 mol reagent reacting per active site [27]. Taking into account that borate buffer was used [26] and the specificity of the reagent [9,24,25] it is probable that inactivation resulted from modification of one arginine per active site.

The ATPase activity of TF_1 was rapidly inactivated by Woodward's reagent K (Fig. 2). Inactivation followed pseudo-first-order kinetics for some time,

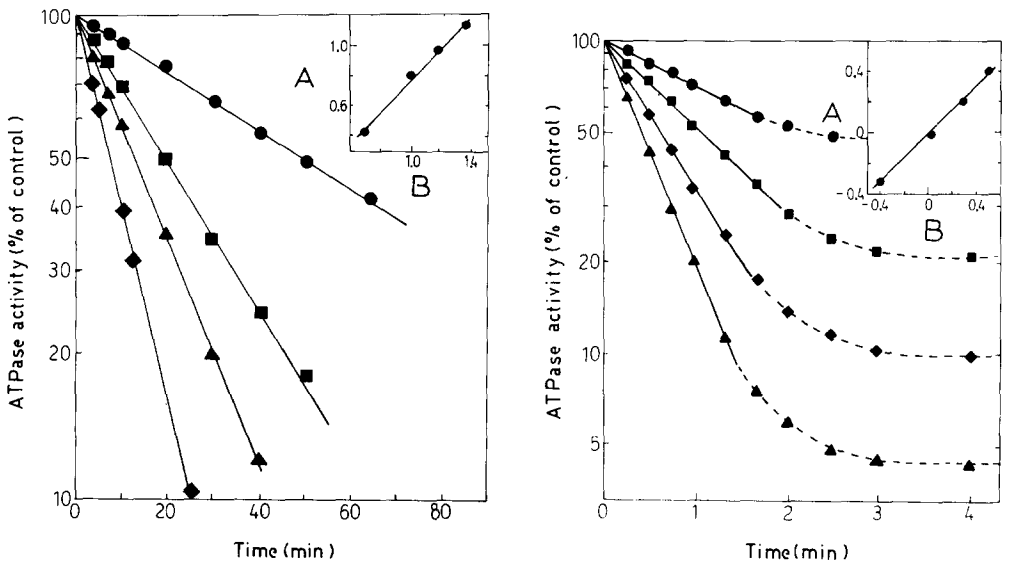

Fig. 1. Inactivation of the ATPase activity of TF_1 by phenylglyoxal. TF_1 was modified by 5 (●——●), 10 (■——■), 15 (▲——▲) and 25 (◆——◆) mM phenylglyoxal. A, log $1/t_{0.5}$; B, log [phenylglyoxal].

Fig. 2. Inactivation of the ATPase activity of TF_1 by Woodward's reagent K. TF_1 was modified by 0.5 (●——●), 1 (■——■), 2 (◆——◆) and 4 (▲——▲) mM Woodward's reagent K. A, log $1/t_{0.5}$; B, log [Woodward's reagent K].

TABLE I

PROTECTION OF TF_1 BY NUCLEOTIDES AGAINST INACTIVATION BY PHENYLGLYOXAL AND WOODWARD'S REAGENT K

Half-times of inactivation ($t_{0.5}$) of TF_1 by 10 mM phenylglyoxal or 1 mM Woodward's reagent K are reported. The enzyme was preincubated for 5 min with the nucleotides stated. The numerals in parenthesis indicate percentage increase of $t_{0.5}$ by added nucleotides. AMP-PNP, adenyl imidodiphosphate.

Addition during preincubation (mM)	ATPase activity $t_{0.5}$ (min)	
	Phenylglyoxal	Woodward's reagent K
None	18	1.0
ATP (0.1)	30 (70)	1.9 (90)
ATP (1)	45 (150)	2.4 (140)
ATP (10)	55 (200)	2.8 (180)
ADP (0.1)	28 (55)	2.1 (110)
ADP (1)	50 (180)	3.1 (210)
ADP (10)	70 (290)	4.0 (300)
GDP (1)	29 (61)	1.4 (40)
IDP (1)	30 (67)	1.5 (50)
GTP (1)	27 (50)	—
AMP-PNP (0.1)	29 (61)	—
CTP (1)	18 (0)	1.0 (0)

after which disappearance of reagent by hydrolysis became noticeable. Concencentrations of the reagent higher than 4 mM gave complete inactivation. The order of reaction with respect to Woodward's reagent K was 0.9 (inset Fig. 2), suggesting that incubation caused 1 mol of reagent to modify one carboxyl group per active site.

The presence of ADP or ATP during preincubation of TF_1 with either phenylglyoxal or Woodward's reagent K afforded considerable protection against inactivation (Table I). GDP and IDP protected but were less effective, whilst CTP and P_i (10 mM, not shown) did not protect at all. $MgCl_2$ neither protected nor increased protection by adenine nucleotides (not shown). Protection afforded to TF_1 against modification by both reagents was remarkably similar for each nucleotide assayed (see percentage of increase in $t_{0.5}$ in Table I).

Arginine residues have been shown to play a role in anionic binding sites of proteins [9] and particularly in the adenine nucleotide binding site of several enzymes [10—12]. The complete inactivation of the ATPase activity of TF_1 by phenylglyoxal (Fig. 1) and the protection afforded by the substrate (Table I) are in agreement with similar findings in other ATPases, namely in the soluble coupling factors from chloroplasts [11], mitochondria [10] and *R. rubrum* chromatophores [12], and suggest the presence of an essential arginine in the catalytic site of TF_1 although we have no direct evidence of this.

Woodward's reagent K is more stable but reacts more slowly at pH 6 than at pH 8. The reagent also inactivated the ATPase activity of TF_1 at pH 6 (data not shown) adenine nucleotides also protected it against inactivation. The reagent has been used to modify essential carboxyl groups in chloroplast coupling factor 1 [17] and in other enzymes such as phosphoglycerate kinase [16]. Both were protected against inactivation by adenine nucleotides. Although five carboxyl groups of the kinase were modified, only one appears to be essential.

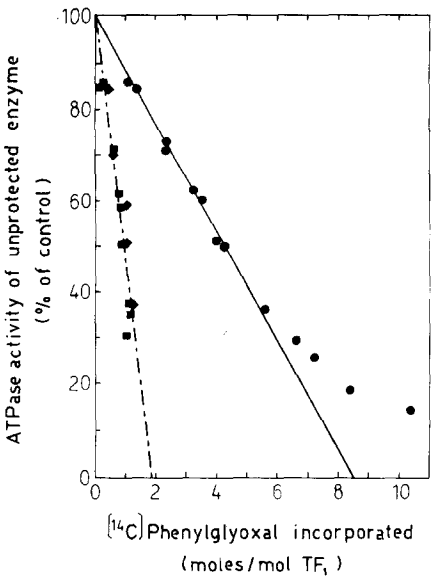

Fig. 3. Incorporation of [^{14}C]phenylglyoxal into TF_1. Modification was carried out in 100 mM *N*-ethyl morpholine/acetate (pH 8) in the absence of (●) or in the presence of 20 mM ATP (■) or ADP (◆). The difference in incorporation between samples without and with adenine nucleotides at corresponding time points is shown in the dashed curve (■,◆).

Attempts to determine the number of groups modified in TF_1 were unsuccessful.

Adenine nucleotides can bind α and β subunits of TF_1, whilst CTP binds to α but not to β subunits [28]. Since the former protected TF_1 against phenylglyoxal and Woodward's reagent K (Table I) while the latter did not, it is possible that the functional arginine and carboxyl residues are on the adenine nucleotide binding sites on β subunits.

To determine how many arginines of TF_1 were modified during inactivation of its ATPase activity [^{14}C]phenylglyoxal was used. Fig. 3 shows that the incorporation of [^{14}C]phenylglyoxal in *N*-ethyl morpholine/acetate buffer into TF_1 was linear with ATPase inactivation up to 65% inhibition. Extrapolation to 100% inactivation gave 8—9 mol [^{14}C]phenylglyoxal incorporated per mol of TF_1. When modification of TF_1 was carried out in the presence of 25 mM ATP or ADP considerable protection of the ATPase activity was obtained and less radioactivity was incorporated into TF_1.

When the differences between the [^{14}C]phenylglyoxal incorporated in the absence and in the presence of ATP or ADP were plotted a straight line was obtained that extrapolates (Fig. 3) to a value of 2 mol per mol of TF_1.

CTP, which did not protect the ATPase activity of TF_1 against modification by phenylglyoxal (Table I), prevented the incorporation of [^{14}C]phenylglyoxal into an arginine residue from the residue that was protected by adenine nucleotides (results not shown). This suggests that there may be an arginine involved in the binding site of CTP in the α subunit [28].

It is interesting to note that, since it is known that 2 mol phenylglyoxal react with one arginine residue in the absence of borate buffer [24], only four or

five arginine residues of the 204 which are present in TF_1 [8] were modified when the ATPase was inactivated (Fig. 3). Moreover, the results of Fig. 3 suggest that only one arginine residue per mol TF_1 is protected by ADP or ATP and is responsible of the inhibition observed. Essentially the same result has been obtained with the chloroplast coupling factor 1 (Viale et al., unpublished results). Kohlbrenner and Cross [29] have recently reported that efrapeptin, a competitive inhibitor of the mitochondrial ATPase, protects a single rapidly reacting arginine of the enzyme. Inactivation of TF_1 by modification of only one arginine per mol may be explained by the alternating site mechanism of ATP synthesis [30].

Acknowledgements

This work was supported by grants from the Consejo Nacional de Investigaciones Científicas y Técnicas, Argentina. José Luis Arana is a Fellow and Rubén Héctor Vallejos a member of the Investigator Career of the same institution.

References

1 Yoshida, M., Sone, N., Hirata, H. and Kagawa, Y. (1975) J. Biol. Chem. 250, 7910—7916
2 Kagawa, Y. (1978) Biochim. Biophys. Acta 505, 45—93
3 Yoshida, M., Sone, N., Hirata, H. and Kagawa, Y. (1977) J. Biol. Chem. 252, 3480—3485
4 Yoshida, M., Okamoto, H., Sone, N., Hirata, H. and Kagawa, Y. (1977) Proc. Natl. Acad. Sci. U.S.A. 74, 936—940
5 Senior, A.E. (1973) Biochim. Biophys. Acta 301, 249—277
6 Senior, A.E. (1975) Biochemistry 14, 660—664
7 Ravizzini, R.A., Andreo, C.S. and Vallejos, R.H. (1980) Biochim. Biophys. Acta 591, 135—141
8 Yoshida, M., Sone, N., Hirata, H., Kagawa, Y. and Ui, N. (1979) J. Biol. Chem. 254, 9525—9533
9 Riordan, J.F. (1979) Mol. Cell. Biochem. 26, 71—92
10 Marcus, F., Schuster, S.M. and Lardy, H.A. (1976) J. Biol. Chem. 251, 1775—1780
11 Andreo, C.S. and Vallejos, R.H. (1977) FEBS Lett. 78, 207—210
12 Vallejos, R.H., Lescano, W.I.M. and Lucero, H.A. (1978) Arch. Biochem. Biophys. 190, 578—584
13 Esch, S.F. and Allison, W.S. (1978) J. Biol. Chem. 253, 6100—6106
14 Pai, E.F., Sachsenheimer, W., Schirmer, R.H. and Schulz, G.E. (1977) J. Mol. Biol. 114, 37—45
15 Vallejos, R.H., Viale, A. and Andreo, C.S. (1977) FEBS Lett. 84, 304—308
16 Brake, A.J. and Weber, B.H. (1974) J. Biol. Chem. 249, 5452—5457
17 Arana, J.L. and Vallejos, R.H. (1980) FEBS Lett., 113, 319—322
18 Gepshtein, A. and Carmeli, C. (1974) Eur. J. Biochem. 44, 593—602
19 Pougeois, R., Sastre, M. and Vignais, P.V. (1978) Biochemistry 17, 3018—3023
20 Pougeois, R., Sastre, M. and Vignais, P.V. (1979) Biochemistry 18, 1408—1412
21 Sumner, J.B. (1944) Science 100, 413—414
22 Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265—275
23 Penefsky, H.S. (1977) J. Biol. Chem. 252, 2891—2899
24 Takahashi, K. (1968) J. Biol. Chem. 243, 6171—6179
25 Takahashi, K. (1977) J. Biochem. 81, 403—414
26 Werner, M.M., Moldovan, M. and Sokolovsky, M. (1975) Eur. J. Biochem. 53, 207—216
27 Levy, H.M., Leber, P.D. and Ryan, E.M. (1963) J. Biol. Chem. 239, 3654—3659
28 Ohta, S., Tsuboi, M., Oshima, T., Yoshida, M. and Kagawa,Y. (1980) J. Biochem. (Tokyo), in the press
29 Kohlbrenner, W.E. and Cross, R.L. (1978) J. Biol. Chem. 253, 7609—7611
30 Hackney, D.D. and Boyer, P.D. (1978) J. Biol. Chem. 253, 3164—3170