SPECIFICITY OF BOVINE HEART PROTEIN KINASE FOR THE Δ-STEREOISOMER OF THE METAL–ATP COMPLEX

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1. Introduction

We have shown, by kinetic and metal-binding studies that the catalytic subunit of bovine heart protein kinase tightly binds 2 divalent cations only in the presence of nucleotides such as ATP [1]. The data were interpreted to indicate that 1 of the 2 divalent cations interacts solely with the enzyme-bound ATP forming the active ternary enzyme–ATP–M^{2+} complex. As determined by NMR, the other, somewhat less tightly-bound divalent cation, bridges the enzyme to the triphosphate chain of the metal–ATP complex, forming a highly-inhibited

quaternary enzyme–ATP–M^{2+} complex [2].

M^{2+}

The precise location of and stereochemistry about the activating metal ion on the enzyme-bound ATP were not determined.

Stable ATP complexes of the trivalent metal ions Cr(III) and Co(III) when functional as substrates provide an independent and direct means of determining the chelate structure and absolute stereochemistry of the active metal–ATP complex [3–5]. Such studies with hexokinase [6,7], phosphoribosyl pyrophosphate synthetase [8] and pyruvate kinase [9] have shown that the β,γ bidentate metal–ATP chelate is the active substrate, although the rates with these substrates are in general very low. Further, due to the chirality at the β-phosphorus of the bidentate complexes, two stereoisomers with absolute configurations denoated as Λ and Δ [5] are possible (fig.1). While both stereoisomers may bind at the active site of an enzyme, only one configuration is expected to be preferred as a substrate. Making use of the known absolute stereochemistry of such complexes [5] it has been shown that the Λ-isomer of β,γ bidentate $Co(NH_3)_4$–ATP is the substrate for hexokinase [7] while the Δ-isomers of $Co(NH_3)_4$–ATP and of Cr–ATP are substrates for phosphoribosyl pyrophosphate synthetase [8] and pyruvate kinase [9], respectively. The Δ- and Λ-stereoisomers of $Co(NH_3)_4$–ATP have been shown to bind to the catalytic subunit of protein kinase with equal affinity [1]. In the present work the Δ-isomer of β,γ bidentate $Co(NH_3)_4$–ATP is shown to be the preferred substrate.

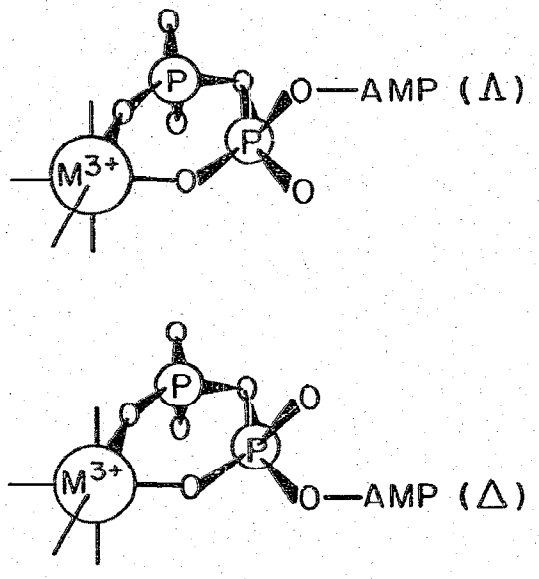

Fig.1. The Δ- and Λ-stereoisomers of the β,γ bidentate metal–ATP complex [3–5].

2. Materials and methods

2.1. *Materials*

The catalytic subunit of protein kinase from bovine heart was purified to homogeneity as in [1,10]. β,γ Bidentate $Co(NH_3)_4$–ATP was prepared by the method in [3] and the Δ-stereoisomer was separated from the racemic mixture with hexokinase as in [7,8]. Pyruvate kinase was obtained from Sigma. Lactate dehydrogenase and adenylate kinase were obtained from Boehringer Mannheim GmbH. β-NADH was obtained from Sigma.

2.2. *Methods*

The catalytic activity of protein kinase was assayed using the heptapeptide substrate Leu–Arg–Arg–Ala–Ser–Leu–Gly as in [1]. The amount of free ATP in the $Co(NH_3)_4$–ATP complex was found to be $< 1\%$ by the coupled hexokinase and glucose-6-phosphate dehydrogenase reactions [11]. The activity of $Co(NH_3)_4$–ATP as a substrate for protein kinase was monitored by 3 independent methods:

(i) The formation of ADP due to the decomposition of the unstable reaction product ADP–$Co(NH_3)_4$–P–peptide;

(ii) The effect of phosphorylation on the ^1H NMR spectrum of the synthetic heptapeptide substrate;

(iii) The change in the circular dichroism spectrum of racemic $Co(NH_3)_4$–ATP as one stereoisomer was used [7,8].

In addition the reaction product was treated with either 0.01 M HCl or 1% acetic acid for 10 h at 22°C in order to remove the Co(III). The phosphopeptide component of the product was identified by its characteristic position on elution from a Sephadex G-10 column (45 × 0.75 cm) and by its electrophoretic mobility in high-voltage paper electrophoresis, at pH 3.5 and 6.5, using the authentic phosphopeptide as a standard in both cases. The phosphopeptide standard was obtained by enzymatic phosphorylation of the peptide with Mg–ATP.

The ADP assay was carried out using the pyruvate kinase and lactic dehydrogenase reactions [12]. The assay mixture (0.6–1.0 ml) contained 83 mM Tris–Cl at pH 7.5, 7 mM $MgCl_2$, 50 mM KCl, 3.3 mM phosphoenol pyruvate and 0.1 mg/ml β-NADH. Formation of AMP, due to spontaneous ADP hydrolysis after prolonged incubations, was also determined using adenylate kinase in the presence of ATP [12], followed by ADP assay. All the incubation mixtures contained, apart from the reacting materials, 50 mM Tris–Cl at pH 7.5, 150 mM KCl and 0.1 mM dithiothreitol. Aliquots of the incubation mixtures were diluted into the ADP assay mixtures at several time intervals. ^1H NMR spectra were recorded on a Bruker WH 180/360 spectrometer operating at 360 MHz in the Fourier transform mode. The NMR solutions were prepared with 99.7% D_2O and contained deuterated Tris–Cl (d11) at pD 7.5. Circular dichroism measurements were performed on a Jasco J-41 spectropolarimeter equipped with a data processor using a 2 cm pathlength. All kinetic and spectroscopic studies were done at 25°C.

3. Results and discussion

If protein kinase were to utilize an isomer of $Co(NH_3)_4$–ATP as a substrate, the reaction catalyzed might be as follows:

$$Co(NH_3)_4\text{–ATP} + \text{peptide} \xrightarrow{E} ADP\text{–}Co(NH_3)_4\text{–P–peptide} \quad (1)$$

In subsequent, uncatalyzed steps the unstable Co(III)-bridged product could decompose:

$$ADP\text{–}Co(NH_3)_4\text{–}P\text{–}peptide \longrightarrow \longrightarrow$$

$$ADP + P\text{–}peptide + CO^{3+}(NH_3)_4(H_2O)_2 \qquad (2)$$

ultimately yielding free ADP which can be monitored quantitatively.

To determine whether $Co(NH_3)_4$–ATP can function as a substrate of protein kinase mixtures of enzyme, $Co(NH_3)_4$–ATP (either the racemic mixture or the resolved Δ-isomer), peptide and Mn^{2+} were incubated for several periods ranging from 0.6–48 h. The amount of ADP formed as well as the amount of AMP formed after prolonged incubation were determined as in section 2.2. The results, summarized in table 1, clearly demonstrate that approximately half of the racemic mixture (∿44%) and the major part of the resolved Δ-isomer (∿76%) were utilized in the presence of protein kinase. Since both isomers of $Co(NH_3)_4$–ATP bind to the enzyme with similar affinities [1] this implies that the Δ-isomer is preferred as a substrate by protein kinase. The identification of the phosphopeptide product was established by its comigration with the authentic phosphopeptide in gel filtration with Sephadex G-10 and on high-voltage paper electrophoresis.

The present finding that $Co(NH_3)_4$–ATP is used as a substrate by protein kinase even in the absence of additional metal ions (cf. table 1) supports our previous suggestion that the active protein kinase complex is an enzyme–nucleotide–metal complex. As found with hexokinase [6,7], phosphoribosyl pyrophosphate synthetase [8] and pyruvate kinase [9], the rate with the stable metal–ATP complex is,

Table 1
Substrate activity of $Co(NH_3)_4$–ATP in protein kinase reaction

Exp.	Initial concentrations of species				Incubation time (h)	Fraction of reacting $Co(NH_3)_4$–ATP (%)[b]
	Enzyme (μM)	$Co(NH_3)_4$–ATP[a] (mM)	Peptide (mg/ml)	Mn^{2+} (mM)		
I	–	3.9 (*R*)	–	–	0.6	0.3
					24	2.0
					48	3.5
II[c]	60	3.9 (*R*)	–	–	0.6	1.2
					1.1	2.0
					6.8	3.3
III	60	3.9 (*R*)	4.1		0.6	21
					1.1	24
					6.8	36
					24.5	45
	60	2.3 (*R*)	4.1		24	44
					48	45
IV	60	3.9 (*R*)	4.1	2.0	0.6	22
					1.1	24
					6.8	34
					24.5	41
V	60	3.8 (Δ)	4.1	–	0.6	40
					2.5	44
					19.3	70
VI	60	2.3 (Δ)	4.1	–	24	77
					48	75

[a] *R* and Δ denote the racemic mixture and the resolved Δ isomer, respectively

[b] Total amount of ADP and AMP formed relative to the initial $Co(NH_3)_4$–ATP concentration

[c] The small amount of activity in this experiment may reflect enzyme-catalyzed hydrolysis of $Co(NH_3)_4$–ATP as found with Mg–ATP [15]

however, very low. Thus while the V_{max} is 2000 min^{-1} with Mg–ATP and 510 min^{-1} with Mn–ATP [1], it is only 0.5 min^{-1} with $Co(NH_3)_4$–ATP*. The rapid decrease in the reaction rate with time observed for incubation periods > 1 h, which is manifested in particular with the Δ-isomer, may be attributed to potent inhibition by the products. The ADP product formed in reaction (2) binds to protein kinase ~4-fold tighter than $Co(NH_3)_4$–ATP and its formation in large amounts, occasionally exceeding the concentration of the substrate, will thus strongly inhibit the reaction.

The ^1H NMR spectra of the peptide substrate in the presence of protein kinase (135 μM), racemic $Co(NH_3)_4$–ATP (2.2 mM), and varying amounts of Mn^{2+} (0–140 μM), were taken at several time intervals. It was found that the intensity of C_β–H_2 resonance of the serine residue at 3.74 ppm from DSS decreased with time, while simultaneously a new resonance was increasing in magnitude 0.07 ppm downfield. These observations provide direct evidence for the phosphorylation of the serine residue by protein kinase in the presence of $Co(NH_3)_4$–ATP. The chemical shift of the new resonance was 0.11 ppm upfield from that found with the phosphopeptide prepared with Mg–ATP indicating that Co(III) remains coordinated to the phosphopeptide. This point is supported by the observed changes in the chemical shift of the free phosphopeptide upon heating with $Co^{3+}(NH_3)_4(H_2O)_2NO_3$ at 80°C for 10 min, and by the residual pink color of the separated phosphopeptide product from $Co(NH_3)_4$–ATP. From the integrated intensities of the serine resonances, the reaction rate was found to be comparable to that obtained by the ADP-assay methods suggesting that the rate of decomposition of the Co(III)-bridged product (see reaction (2)) exceeds the rate of formation (reaction (1)).

In order to further establish the stereochemistry of the substrate isomer of $Co(NH_3)_4$–ATP, a CD spectrum was taken of a 48 h incubation mixture which initially contained the racemic mixture. The CD spectrum of a racemic mixture alone (fig.2A) shows both positive and negative ellipticity, in the region of absorption of the Co(III) chromophore, due to imperfect cancellation of the ellipticities of the Δ- and Λ-isomers [3,7,8]. The CD spectrum of the reaction mixture shown in fig.2B manifests only positive ellipticity, which characterizes the inactive Λ-isomer of $Co(NH_3)_4$–ATP, thus confirming that the Δ-isomer was utilized as the preferred substrate.

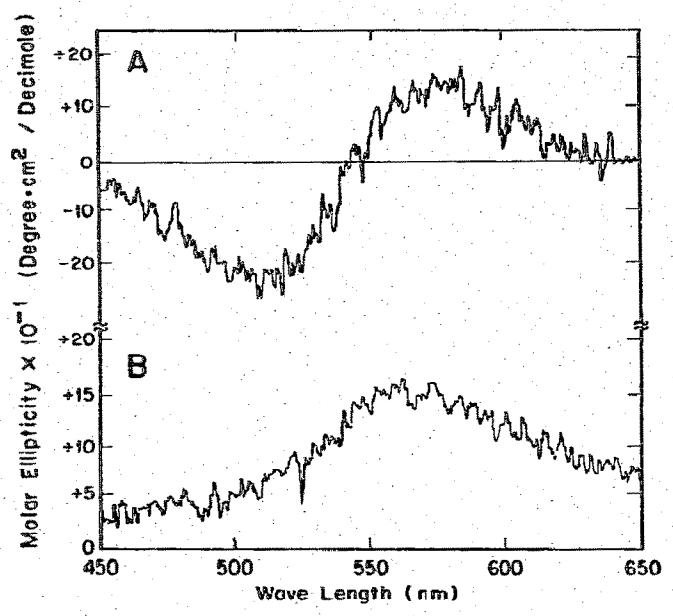

Fig.2. Computer averaged CD spectra at 25°C of: (A) racemic mixture of $Co(NH_3)_4$–ATP isomers (4.0 mM); (B) reaction mixture after 48 h incubation with $Co(NH_3)_4$–ATP (initially 2.2 mM racemate), heptapeptide substrate (5.5 mM) and protein kinase (135 μM catalytic subunit). Other components present were: deuterated Tris–Cl pD 7.5 (10 mM); $MnCl_2$ (140 μM); KCl (150 mM). (A) is the difference spectrum obtained by subtracting 4 scans of water from 4 scans of the sample. (B) is the difference spectrum obtained by subtracting 16 scans of buffer solution from 16 scans of the reaction mixture.

* With Mg^{2+} as the activator, the catalytic subunit of protein kinase was found to utilize the A-isomer of ATPβS at a rate comparable to that of ATP, and the B-isomer at least an order of magnitude more slowly [13]. Since the Mg^{2+} complex of the A-isomer of ATPβS is thought to have the Δ-configuration [14], this observation provides further evidence for the utilization of the Δ-isomer with a highly reactive metal–ATP complex

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References

[1] Armstrong, R. N., Kondo, H., Granot, J., Kaiser, E. T. and Mildvan, A. S. (1979) Biochemistry 18, 1230–1238.
[2] Granot, J., Armstrong, R. N., Kondo, H., Kaiser, E. T. and Mildvan, A. S. (1979) Biochemistry, in press.
[3] Cornelius, R. D., Hart, P. A. and Cleland, W. W. (1977) Inorg. Chem. 16, 2799–2805.
[4] Cleland, W. W. and Mildvan, A. S. (1979) Adv. Inorg. Biochem. 1, 163–191.
[5] Merritt, E. A., Sundaralingam, M., Cornelius, R. D. and Cleland, W. W. (1978) Biochemistry 17, 3274–3278.
[6] Danenberg, K. D. and Cleland, W. W. (1975) Biochemistry 14, 28–39.
[7] Cornelius, R. D. and Cleland, W. W. (1978) Biochemistry 17, 3279–3286.
[8] Li, T. M., Mildvan, A. S. and Switzer, R. L. (1978) J. Biol. Chem. 253, 3918–3923.
[9] Dunaway-Mariano, D., Benovic, J., Cleland, W. W., Gupta, R. K. and Mildvan, A. S. (1979) submitted.
[10] Demaille, J. G., Peters, K. A. and Fischer, E. H. (1977) Biochemistry 16, 3080–3086.
[11] Lamprecht, W. and Trautschild, I. (1965) in: Methods of Enzymatic Analysis (Bergmeyer, H. U. ed) pp. 543–551, Academic Press, New York.
[12] Adam, H. (1965) in: Methods of Enzymatic Analysis (Bergmeyer, H. U. ed) pp. 573–577, Academic Press, New York.
[13] Bolen, D. W., Stingelin, jr and Kaiser, E. T. (1979) unpublished results.
[14] Jaffe, E. and Cohn, M. (1978) J. Biol. Chem. 253, 4823–4825.
[15] Armstrong, R. N., Kondo, H. and Kaiser, E. T. (1979) Proc. Natl. Acad. Sci. USA 76, 722–725.