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Immobilization of ATP on Bovine β-Caseins by Using Transglutaminase

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효소법에 의한 ATP의 Bovine β-Casein에의 고정화

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ABSTRACT

ATP analogs were immobilized on bovine caseins by the action of transglutaminase. The ATP analogs immobilized on the caseins were enzymatically active and interconverted by kinases. The immobilized ATP was dephosphorylated to the corresponding ADP by hexokinase and rephosphorylated to the ATP in solid form by acetate kinase. Under the conditions chosen, about 55% of the immobilized ATP was dephosphorylated and about 80% of the resulted ADP was rephosphorylated. Bovine β-casein was more useful than α_{sf}-casein as a carrier and C^8-substituted ATP analognwas more effective than N^6-substituted one in immobilization. Michaelis constant of C^8-substituted ATP analog immobilized on β-casein was similar to that of free form of ATP and that of ATP analog. The immobilized ATP was much more stable than free ATP and its analog, while maximum velocity was reduced to 37% of the free ATP analog. The immobilized ATP was recovered almost completely by calcium precipitation.

INTRODUCTION

The last decade have been a remarkable upsurge of interest in the practical application of enzymes in the industrial, analytical, and biomedical fields. These applications of enzymes have been enlarged by the development of the immobilization methods of enzymes to permit reuse and recovery. But industrial applications of enzymes have been restricted so far to systems operating without coenzymes. Future generation, not yet in practical use, will include those enzymes requiring coenzymes for catalytic activity. For example, of the 2,000 or so different enzymes to which a specific number has been assigned, over one-third require one of the five principal adenine nucleotide-containing coenzymes (NAD^+, $NADP^+$, ATP, FAD, and Coenzyme A)for catalytic activity. Therefore, some consideration for practical application of the enzymes must include discussions of problems associated with readily dissociable coenzymes.

Because coenzymes are expensive, their economic use would necessitate methods to retain them in the reaction mixture and to regenerate them for following reactions. Coenzyme-retention may be achieved by immobilization to a carrier matrix or entrapment of the immobilized coenzyme within a limiting membrane or polymer matrix. To achieve this aim, a functional group suitable for covalent

atachment to a solid support must be chemically introduced into the coenzyme, ideally with a spacer molecule inserted between the matrix backbone and coenzyme to alleviate steric hindrance with enzyme binding(1−3). The most desirable position to introduct a spacer(for example, $-NH-CH_2-COO^-$, $-NH-CH_2-CONH-(CH_2)_6-NH_3^+$, $-NH-CO-NH-(CH_2)_6-NH_3^+$, or $-NH-(CH_2)_6-NH_3^+$) into adenine nucleotide molecules appears to be the amino group at the N^6 site or C^8 site on the adenine nucleus with the aim of maintaining the coenzymatic activities of the derivatives (4−8). Macromolecular derivatives of NAD^+, and ATP have been prepared as reusable coenzymes, but their coenzyme activities are generally lower than those of native ones(9). The cost of preparing a macromolecular derivative of the coenzyme can be minimized if the synthesis can be carried out by simple but efficient method.

The established strategy for the synthesis of high-molecular weight coenzyme derivative is to synthesize a reactive and well-defined coenzymatically active analog, followed by the covalent attachment(10−15), alkylation (16) of this analog to a suitable water-soluble macromolecule. The main disadvantage of this approach is a low yield, due to many steps in the synthesis and purification of coenzyme analogs. Others(17, 18) synthesized coenzymatically active macromolecular derivatives of NAD by copolymerizations. The main disadvantage of these simple methods is the occurrence of side reactions, which results in nonuniform macromolecular coenzyme derivatives.

In the previous work (19), bovine β-casein was found to be a good support to bind with NAD^+ analog by the action of transglutaminase. Transglutaminase catalyzes an acyl-transfer reaction in wich γ-carboxamide groups of peptide-bound glutaminyl resudues are acyl donors. We now report a simple procedure for immobilization of ATP on bovine β-casein by the same enzymatic method, because ATP has the same adenine nucleus as NAD^+.

MATERIALS AND METHODS

Enzymes and coenzymes

Transglutaminase (glutaminyl-peptide γ-glutamyl transferase: EC 2.3.1.13), hexokinase, acetate kinase, ATP, and ATP analogs (8−(6−aminohexyl) aminoadenosine 5'-triphosphate and N^6−[6-aminohexyl)−carbamoylmethyl]−ATP were perchased from Sigma(USA).

Caseins

Whole acid casein was prepared from fresh skim milk by adjusting pH to 4.7, as described by Hipp et al. (20). The urea method by Hipp et al.(21) was used to fractionate α-and β-casein from the whole acid casein. β-Casein fraction was purified by repeating the fractionation procedure three times and dialyzed against a large volume of distilled water to remove any traces of urea. α_{S1}-Casein was isolated from α-casein by the method of Tsugo and Yamauchi (22).

Immobilization of ATP on caseins

Immobilization was carried out basically according to the method by Yoshikawa et al.(23). Transglutaminase at concentration of 180㎍/ml (2.5 units/ml) was added to 10mM Tris-HCl buffer, pH 7.5, containing 5mM $CaCl_2$, 100mM dithiothreitol, 1.2㎎/ml casein, and 1mM ATP or ATP analog. The reaction mixture was incubated at 37℃ for 2hr. Reaction was terminated by adding EDTA at a final concentration of 36mM. The reaction mixtures were dialysed against 100mM triethanolamine-HCl buffer, pH 7.5, to remove free ATP or its analog. The dialysed samples, containing free ATP or ATP analog bound to caseins were used in all subsequent studies.

Determination of ATP and its analog immobilized on caseins

The number of ATP analog molecules bound to caseins was determined by measuring organic phosphorus in the dialysed samples according to the method of Bartlett (24). The number of bound ATP molecules was calculated by subtracting organic phosphorus contents of caseins in controls from those of caseins subjected to the transglutaminase-catalyzing reaction.

Enzymatic interconversion of immobilized ATP and the corresponding ADP

Enzymatic conversion of immobilized ATP to the corresponding ADP

Hexokinase (2 units/ml) in 100mM Tirs-HCl buffer, pH 7.5, containing glucose (100mM) and Mg^{2+}(5mM) was incubated at 25℃ for 15 min with

the immobilized ATP(1.2mg / ml as casein). The amount of residual ATP activity was determined with ATP kit.

Precipitation of immobilized coenzyme with calcium

To the reaction mixture mentioned just above 1M calcium chloride was added to give final concentration of 36mM. It was centrifugated at 4,500rpm for 20 min. The precipitate was solubilized in 1ml of 50mM EDTA.

Enzymatic rephosphorylation of the corresponding ADP

Dissolved solution of the precipitated casein which contained ATP and ADP in solid phase, was added to a solution containing acetyl phosphate (20mM), Mg^{2+} (3mM) and acetate kinase (7 units / ml) in 100mM Tris-HCl buffer, pH 7.5 and incubated at 25℃ for 15 min. The ATP activity was determined with ATP kit.

Protein concentration

Protein concentrations were determined by the method of Lowry et al.(25).

RESULTS AND DISCUSSION

Immobilization of ATP on bovine caseins

An amino group of a spacer introduced in adenine nucleus of ATP was appeared to be a good acyl acceptor in the transglutaminase reaction from the fact that primary amino groups in a variety of compounds act as acyl acceptors with the subsequent formation of monosubstituted γ-amides of peptide-bound glutamic acid (26). Two ATP analogs, ATP-C^8-$NH(CH_2)_6NH_2$ (symbolized as C^8-ATP) which is substituted at the cyclic C^8 site of the adenine moiety and ATP-N^6-$CH_2CONH(CH_2)_6NH_2$(symbolized as N^6-ATP)which is substituted at the exocyclic N^6 site of the adenine moiety, were used to covalently bind to bovine caseins through the aminohexyl group by action of transglutaminase, shown below.

$$(\text{Casein})-\text{Glu}-\overset{\overset{O}{\|}}{C}-NH_2+NH_2\sim(\text{ATP}) \xrightarrow{\text{Transglutaminase}} (\text{Casein})-\text{Glu}-\overset{\overset{O}{\|}}{C}-NH\sim(\text{ATP})+NH_3$$

Fig. 1 shows UV absorption spectra of ATP analog, C^8-ATP and β-casein mixture and of ATP analog which was subjected to the transglutaminase reaction, followed by intensive dialysis to remove free C^8-ATP. The UV

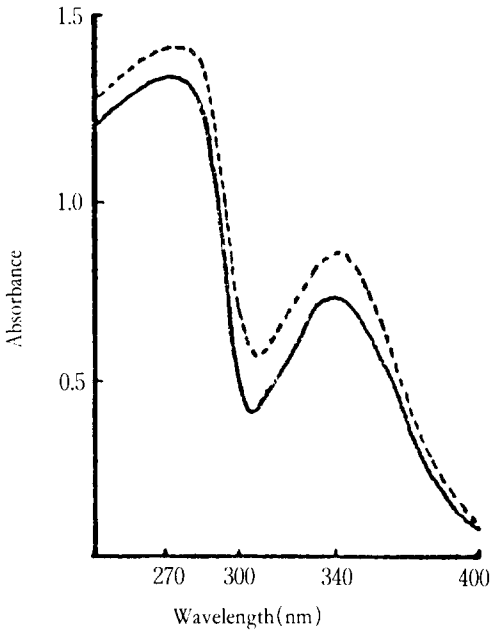


Fig. 1. UV absorption spectra of ATP analog (C^8-ATP)and β-casein mixture(……) and of ATP analog(—) subjected to transglutaminase reaction in the presence of β-casein.

spectrum of C^8-ATP · The UV spectrum of C^8-ATP · β-casein conjugate subjected to the transglutaminase reaction was found to be similar to that of C^8-ATP and β-casein mixture. This spectral similarity seems to be attributable to ATP analog bound to β-casein because the absorption maximum of ATP is at 340 nm. The spectrum of N^6-ATP · β-casein conjugate was similar to that of N^6-ATP and β-casein mixture and same tendency was found in the case of α_{S1}-casein(Data not shown).

When we compared the absorption spectrum of β-casein to that of β-casein subjected to the transglutaminase reaction in the absence of ATP analog (Fig. 2), little difference in absorbancy at 280 nm between them and no spectral shift was observed. This indicates no contribution of transglutaminase-catalysed polymerization of β-casein to spectral change. Thus spectral change surely shows binding of the ATP analogs to β-casein.

The we wanted to know whether or not the ATP analogs immobilized on caseins were coenzymatically active. Caseins subjected to the transglutaminase reaction was

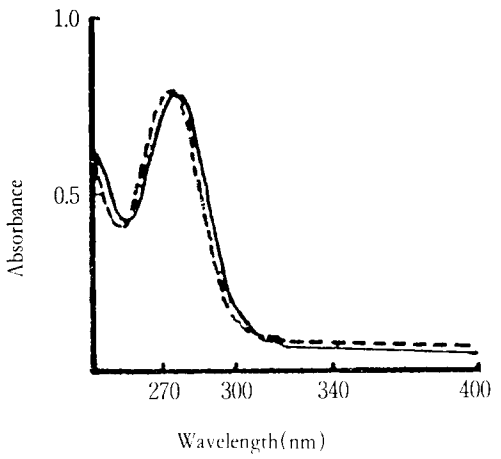


Fig. 2. UV absorption spectra of β-casein (··· ···) and β-casein(——) subjected to the transglutaminase reaction in the absence of ATP analog.

incubated with phosphoglycerate kinase and 3—phospho glycerate, followed by adding glyceraldehyde phosphate dehydrogenase and NADH. Decrease in absorbance at 340 nm which results when NADH is oxidized to NAD^+ was observed, showing that ATP is coenzymatically active.

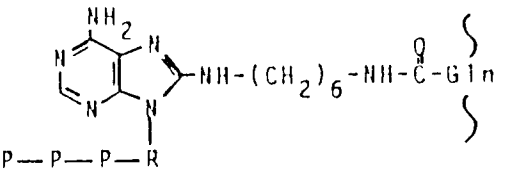
Table 1 shows numbers of ATP analogs immobilized on the caseins. β-Casein was more effective than α_{S1}-casein as substrate. More effectiveness of β casein than α_{S1} casein as substrate would be due to the fact that β-casein has 21 glutaminyl residues per mole while α_{S1} casein has 14 glutaminyl residues. Total moles of the immobilized C^8-ATP analogs were 2.9 and 2.2 per mole of β-casein and α_{S1}-casein, respectively, while those of immobilized N^6-ATP analogs were 2.5 and 2.1 per mole of β-casein and α_{S1}-casein, respectively. At most 2.9 of the 21 glutaminyl residues present in one mole of β casein appear to function as substrates of transglutaminase. It is not clear whether the availability of a glutaminyl residue as substrate depends on the sequence of neighbouring amino acids or on the conformation of the domain containing this residue. ATP analogs are probably bound covalently to caseins through the formation of a γ-glutamylamine bond between the amino group attached to the aliphatic carbon-chain of the adenine moiety and a γ-carboxamide group of glutaminyl residues in the caseins.

Table 1. Coenzymatically active ATP analogs immobillzed on bovine caseins

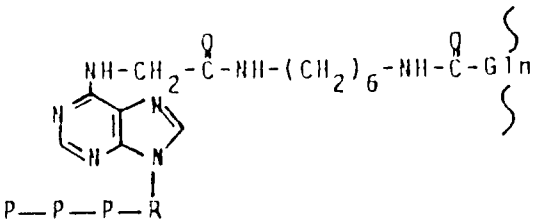
Analogs / Caseins	N^6-ATP	C^8-ATP
β-casein	11%(2.5)	14%(2.9)
α_{S1}-casein	8%(2.1)	11%(2.2)

Coenzymatic activities are presented as percentage against the corresponding free ATP analogs and the numbers shown in parenthesis are moles of ATP mole of casein.

The structures of the casein-bound ATP analogs are probable as shown in Fig. 3. C^8-Substituted ATP analog displayed higher coenzymatic activity comparing to N^6-substituted ATP analog displayed higher coenzymatic activity comparing to N^6 substituted ATP analog. Activity yield of C^8-ATP immobilized on β-casein was about 14 % when the activity was determined with ATP kit. It may be possible to interpret this effect in terms of an electrostatic interaction between the spacer and caseins.



(1)



(2)

Fig. 3. The probable structure of the casein--bound adenine nucleotide coenzyme derivatives.
(1) ATP-C^8-$NH(CH_2)_6NH_2$~casein
(2) ATP-N^6-$CH_2CONH(CH_2)_6NH_2$~casein

Electrostatic repulsion between alkylcarbamoyl group of the spacer of N^6-ATP analog and carboxamide group of casein may hinder the binding.

Enzymatic properties of immobilized ATP

Table 2 shows kinetic parameters of free forms of ATP, ATP analog (C^8 ATP) and the immobilized ATP (C^8 ATP analog · β-casein conjugate). These data were estimated from Lineweaver Burk plots. Apparent affinity of ATP analog for enzyme was not much changed by the immobilization, while maximum rate was reduced to 37% of that of free ATP analog.

Table 2. Coenzymatic properties of C^8-ATP analog immobilized on β-casein

	Km(mM)	Vmax(%)
Free ATP	0.62	120
Free ATP analog	0.65	100
Immobilized ATP	0.87	37

Reaction rates of coenzymes were measured by using ATP kit. Kinetic parameters were obtained from Lineweaver-Burk Plots.

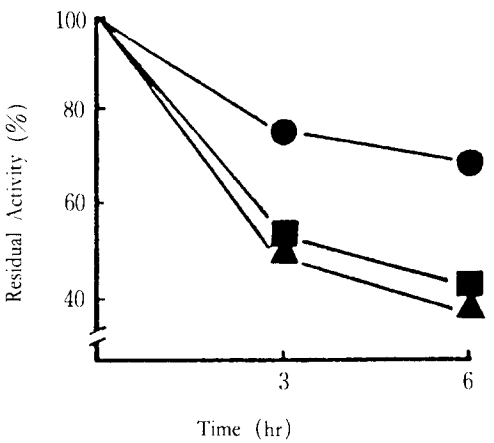


Fig. 4. Stability of C^8-ATP analog immobilized on β-casein. Free ATP(▲), free ATP analog (■), and immobilized ATP (●) were incubated in 100mM Tris-HCl buffer, pH 7.6, at 37℃. During incubation, aliquots were taken out to measure coenzymatic activity.

Fig. 4 shows the effect of immobilization of ATP analog on its stability. When each ATP was incubated at pH 7.6 for 6 hr at 37℃, decrease in their coenzymatic activity was found. The immobilized ATP was much more stable than free form of coenzymes, suggesting that stability of ATP analog was increased by immobilization.

Enzymatic interconversion of the immobilied ATP and the corresponding ADP

Bovine caseins, used here as supports, can be rendered either soluble or insoluble by adjusting calcium concentration or pH. Such an immobilized coenzyme may have significant advantages in practical use. To test the enzymatic conversion, the immobilized ATP was added to glucose with hexokinase and incubated to dephosphorylate to ADP. Under the condition chosen, about 55% of the immobilized ATP was dephosphorylated to the corresponding ADP, based on the decrease in the ATP activity which was recovered in subsequent reaction with acetate kinase.

Calcium chloride was added and the resulted precipitate was solubilized with EDTA, as described in Materials and Methods. It was found that ATP analog immobilized on β-casein was almost completely recovered in the fraction solubilized with EDTA.

Enzymatic rephosphorylation was subsequently accomplished by adding the dephosphorylated β-casein conjugate-containing solution to a solution containing acetyl phosphate and acetate kinase. About 80% of the ADP was rephosphorylated to ATP. Immobolized ATP and its dephosphorylated form were recovered repeatedly by calcium precipitation. This suggests that casein-bound ATP functions as phosphate doner or acceptor for some kinases, and demonstrates a possibility of the immobilized ATP as cofactors useful in ATP-regenerating enzyme reactors. Such a convertible and stable type of coenzyme may have practical advantage.

요 약

Transglutaminase 반응을 이용하여 ATP analog들(C^8 ATP와 N^6-ATP)을 casein에 고정화하였다. 고정화

ATP는 hexokinase에 의해 탈인산화되어 약55%가 ADP 형으로 전환되었으며, 이는 acetate kinase에 의해 역으로 인산화되어 약 80%가 ATP형으로 전환 되었다. β-Casein은 α_{S1}-casein에 비하여 ATP의 고정화 수율이 높았으며 C^8-ATP는 N^6-ATP에 비하여 casein에의 결합이 용이하였는데, 이는 β-casein이 α_{S1}-casein에 비하여 glutamine 잔기의 수가 많으며, N^6-ATP의 경우 이의 alkyl carbamyl기와 casein의 carboxamide기 간의 정전기적 반발에 원인이 있는 것으로 보인다. ATP는 고정화하므로써 안정성이 증대되었으며, 고정화 ATP의 km치는 유리상의 ATP및 ATP analog의 그것과 비슷하였으나, 최대속도는 감소되었다. 고정화 ATP는 반응액에서 calcium 의 첨가로 쉽게 침전되어 용액으로부터 거의 완전히 회수되므로써, 반응액으로부터의 회수분리가 가능한 이점을 가졌다.

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