PROTEIN KINASE ACTIVITY IN CARIOGENIC AND NON-CARIOGENIC ORAL STREPTOCOCCI: ACTIVATION AND INHIBITION BY CYCLIC AMP

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1. Introduction

In recent years, considerable information has been obtained concerning the activity of cyclic AMP-dependent protein kinases in various mammalian tissues and in a number of other animal phyla [1, 2], with this activity being associated both with particulate and soluble cellular fractions [3–9]. However, despite this interest, only two microorganisms, *E. coli* [10] and the acellular slime mold, *Physarum polycephalum* [11], have been shown to contain protein kinases influenced by cyclic AMP. On the one hand, histone phosphorylation by the *E. coli* enzyme was enhanced 3-fold by cyclic AMP, while *P. polycephalum* was shown to contain both a cyclic AMP-activated protein kinase and a cyclic AMP-inhibitable enzyme.

We have previously reported that the oral microbe, *Streptococcus salivarius*, synthesizes cyclic AMP through the action of multiple adenyl cyclases [12]. Subsequent study with highly purified adenyl cyclase III showed that physiological concentrations of various cellular metabolites regulated cyclic AMP formation *in vitro* [13]. Since little is known concerning the effect of cyclic AMP on protein phosphorylation in bacteria, we undertook to determine whether protein kinase activity, as well as cyclic AMP-binding protein, were present in *S. salivarius, S. sanguis* and various strains of the cariogenic genus, *S. mutans*. We wish to report the presence of cyclic AMP-activated protein kinase activity and cyclic AMP-binding protein in these bacteria. Furthermore, since several strains were shown to contain both cyclic AMP-inhibitable as well as cyclic AMP-activated protein kinase activity, these bacteria probably contain at least two protein kinases regulated in a reciprocal fashion.

2. Materials and methods

The procedures for the maintenance, growth and harvesting of the various strains was as described previously for *S. salivarius* [14]. The cells were grown in 0.2% glucose–tryptone–yeast extract broth inoculated with an overnight culture (about 8–10 hr) grown in the same broth with 0.1% glucose. Exponential phase cells were harvested by centrifugation and washed once in Tris-HCl buffer (10 mM, pH 7.0) containing 5 mM mercaptoethanol. Cell extracts were prepared by sonication in a Branson sonifier in the presence of glass beads as outlined previously [12] and centrifuged at 30,000 *g* for 15 min. The supernatant fraction and the pellet, suspended in the Tris-HCl buffer, were dialyzed overnight against the same buffer.

Protein kinase activity was determined according to the method of Reimann et al. [15]. The reaction mixture contained sodium acetate–acetic acid buffer (pH 6.0), 50 mM; EGTA, 0.3 mM; EDTA, 0.2 mM; $MgCl_2$, 10 mM; protein acceptor, 2 mg/ml; [γ-^{32}P]ATP, 0.1 mM ($1-2\times10^5$ cpm/nmole) and enzyme in a final volume of 0.1 ml. NaF and theophylline were eliminated from the assay mix as these compounds inhibited (30–40%) protein kinase activity in our extracts. When added, cyclic AMP was at a concentration of 5 μM. The reaction was always started by the addition of the [γ-^{32}P]ATP and incubation was for 5 min at 37°. The reaction was stopped by the addition of 0.5 ml of 10% TCA along with 0.1 ml of albumin (10 mg/ml). The precipitated protein was centrifuged, reprecipitated, washed and counted in Bray's solution according to the method of Walsh et al.

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Table 1

Protein kinase activity in the dialyzed pellet and supernatant fractions obtained from *S. salivarius, S. sanguis* and various strains of *S. mutans* following sonic breakage and centrifugation at 30,000 *g*.

Organisms	30,000 *g* pellet			30,000 *g* supernatant		
	Without cyclic AMP	With cyclic AMP[a]	Ratio[b]	Without cyclic AMP	With cyclic AMP[a]	Ratio[b]
Boiled control	2.2[c]	2.2	1	2.2	2.2	1
S. salivarius	23.7	99.3	4.2	13.2	13.9	1.1
S. sanguis	13.0	25.4	2.0	9.7	16.9	1.7
S. mutans AHT	26.1	13.3	0.5	16.8	22.7	1.4
S. mutans E-49	16.1	32.9	2.0	37.3	70.3	1.9
S. mutans PS-14	25.6	44.1	1.7	46.3	34.8	0.8
S. mutans OMZ-176	22.2	32.9	1.5	51.9	77.7	1.5
S. mutans 6715	42.0	68.2	1.6	9.9	16.1	1.6

[a] Cyclic AMP concentration was 5 μM.
[b] Ratio of activities with and without cyclic AMP.
[c] pmoles ^{32}P incorporated/mg protein/5 min. Crude histone (0.2 mg) was used as the protein acceptor.

[16]. Under our conditions, reaction velocity was proportional to enzyme concentrations up to 0.2 mg with phosphorylation linear for at least 5 min at that enzyme concentration.

Cyclic AMP-binding protein activity was measured by the method of Anderson et al. [17]. The reaction mixture contained Tris-HCl buffer (pH 7.5), 50 mM; [^{3}H]cyclic AMP, 1 μM ($1-2\times10^6$ cpm/nmole); AMP, 10 mM and enzyme in a final volume of 0.1 ml. Incubation was for 5 min at 0° and protein was precipitated with saturated $(NH_4)_2SO_4$. Nonspecific binding was determined by adding 10 mM unlabelled cyclic AMP to the assay mix [17].

[γ-^{32}P]ATP and [^{3}H]cyclic AMP were purchased from New England Nuclear Corporation. Protamine, α-casein and various histones were purchased from Sigma Chemical Co., while crude histone and albumin were products of General Biochemicals and Mann Research Laboratory, respectively. AMP and cyclic AMP were purchased from Calbiochem. All other reagents were analytical grade.

3. Results

3.1. *Cyclic AMP and protein kinase activity*

The activity of protein kinase in the pellet and supernatant fractions of the non-cariogenic strains, *S. salivarius* and *S. sanguis*, as well as various strains of the cariogenic microbe, *S. mutans*, was tested with and without cyclic AMP following sonic breakage and centrifugation at 30,000 *g*. As shown in table 1, protein kinase activity in the absence of cyclic AMP was higher in the pellet fraction than with the supernatant fraction of *S. salivarius, S. sanguis* 10556 and strains AHT and 6715 of *S. mutans*. On the other hand, the other strains of *S. mutans* (E-49, PS-14 and OMZ-176) showed more activity with the 30,000 *g* supernatant. Of particular interest, however, was the activation of phosphorylation by cyclic AMP which was observed with all preparations, except with the pellet fraction from *S. mutans* AHT and the supernatant fraction from *S. mutans* PS-14. Protein kinase activity in these latter two fractions was inhibited by cyclic AMP. Maximum activation was observed with the *S. salivarius* pellet fraction, with significant activation being observed with both preparations from *S. sanguis* and *S. mutans* E-49.

The effect of the type of protein acceptor on the protein kinase activity in the 30,000 *g* pellet and supernatant fractions obtained from *S. salivarius* was examined in the presence and absence of cyclic AMP. As shown in table 2, protamine was the best phosphate acceptor with either fraction when incubated without cyclic AMP, however, phosphorylation was either unaffected (30,000 *g* pellet) or slightly

Table 2

Protein kinase activity, in the presence of various protein acceptors, of the dialyzed pellet and supernatant fractions from *S. salivarius* following sonic breakage and centrifugation at 30,000 *g*.

Protein acceptor[a]	30,000 *g* pellet			30,000 *g* supernatant		
	Without cyclic AMP	With cyclic AMP[b]	Ratio[c]	Without cyclic AMP	With cyclic AMP[b]	Ratio[c]
Boiled control	3.0[d]	3.0	1	3.0	3.0	1
None	18.6	81.3	4.4	12.3	14.8	1.2
Histone type II[e]	28.0	76.6	2.7	15.2	22.7	1.5
III	18.2	141.6	7.8	7.9	18.0	2.3
IV	19.8	73.7	3.7	12.3	24.0	2.0
V	17.9	72.8	4.1	28.7	37.2	1.3
VI	20.4	65.6	3.2	12.2	28.7	2.4
VII	24.8	50.7	2.0	11.3	20.1	1.8
VIII	17.0	69.2	4.1	12.0	26.9	2.2
Protamine	42.5	42.4	1.0	32.6	27.1	0.8
Albumin	22.8	82.7	3.6	14.5	16.0	1.1
α-Casein	31.5	71.0	2.3	21.3	14.3	0.7

[a] Acceptor concentration was 0.2 mg.
[b] Cyclic AMP concentration was 5 μM.
[c] Ratio of activities with and without cyclic AMP.
[d] pmoles ^{32}P incorporated/mg protein/5 min.
[e] Histone types, II = mix-crude; III = Lysine rich; IV = Arginine rich; V = Lysine rich subgroup f_1; VI = slightly lysine rich subgroup f_{2a}; VII = slightly lysine rich subgroup f_{2b}; VIII = Arginine rich subgroup f_3.

inhibited (30,000 *g* supernatant) by 5 μM cyclic AMP. Of the various types of histone tested, type II was the best acceptor with the pellet fraction when assayed in the absence of cyclic AMP, while significant phosphorylation was also observed with albumin and α-casein. Significantly, the phosphorylation of all of these latter acceptors was activated by cyclic AMP with the maximum activation (8-fold) being observed with histone type III. When the various protein acceptors were incubated with the 30,000 *g* supernatant, maximum activity, both in the presence and absence of cyclic AMP, was observed with histone type V. Again, phosphorylation was activated by 5 μM cyclic AMP to varying degrees, except with casein; the highest stimulation was observed with histone types III, VI and VIII. Table 2 also shows that there was relatively high endogenous accepting activity in both the pellet and supernatant fractions with the pellet activity being activated significantly by cyclic AMP. From a comparison of the pellet and supernatant data, it can be concluded that the cyclic AMP-activated protein kinase in this organism is probably a particulate enzyme. The activating effect of cyclic AMP was observed only with dialyzed fractions due to the presence of endogenous cyclic AMP in undialyzed preparations.

3.2. *Cyclic AMP-binding protein*

It is now well known that protein kinase activity in mammalian tissues is regulated by a protein which binds cyclic AMP [1]. The association of this cyclic AMP-binding protein (regulatory unit) with the catalytic unit of protein kinase makes the latter protein inactive. The binding of cyclic AMP to the regulatory unit causes the dissociation of this inhibitor protein from the catalytic unit which results in the release of the active catalytic unit. In order to determine whether crude extract preparations of the various streptococcal strains contained such a protein, the various dialyzed pellet and supernatant fractions were screened for their cyclic AMP-binding activity. As shown in table 3, the pellet and supernatant fractions from all of the test strains possessed cyclic AMP-binding activity. However, in contrast to the previously

observed protein kinase activity, greater binding activity was observed with the 30,000 *g* supernatant fractions than with the corresponding pellets.

4. Discussion

The cyclic AMP-activated protein kinase activity is widely distributed in eukaryotic cells [1, 2, 11] but in prokaryotic cells its presence has been reported only in *E. coli* [10]. The present evidence supporting the presence of protein kinase and cyclic AMP-binding activity in a number of oral streptococci indicates that these proteins are widely distributed in nature. The protein kinase in *S. salivarius* differs from *E. coli* enzyme with respect to the divalent ion requirement for the activation of protein kinase by cyclic AMP. With *S. salivarius* preparations, we have found that cyclic AMP activation required $MgCl_2$ or $MnCl_2$, with somewhat lower activation (15–25%) being observed with $MnCl_2$. In *E. coli*, cyclic AMP activation was observed only in the presence of $MnCl_2$ [10], while the protein kinases from mammalian tissue are generally active in the presence of $MgCl_2$ [1]. Like the *E. coli* enzyme, cyclic AMP activation of the *S. salivarius* protein kinase was most effective with histone. The inhibition of protein kinase activity by cyclic AMP with preparations from *S. mutans* AHT and PS-14 (table 1) indicates that some strains of oral streptococci contain two types of protein kinases: one activated by cyclic AMP and one inhibited by this nucleotide. Inhibition of protein kinase activity by cyclic AMP has been observed with preparations from *P. polycephalum* [11].

The activity of cyclic AMP-binding protein in the crude preparations of the oral streptococci was somewhat lower than that observed with *E. coli* [17], but almost similar to the activity present in some mammalian tissues [9, 18, 19]. A comparison of tables 1 and 3 indicates that there was little correlation between cyclic AMP-binding activity and protein kinase activity in the streptococcal preparations suggesting that the binding activity may or may not be related to the kinase activity as it is in mammalian systems. It is conceivable that these bacteria may contain more than one cyclic AMP-binding protein, one of which may be similar to the cyclic AMP-receptor protein of *E. coli* which is involved in the regulation of inducible protein synthesis [17]. Further work is in progress to isolate and characterize the protein kinase(s) and cyclic AMP-binding protein(s) in extracts of the oral streptococci.

Table 3
Cyclic AMP-binding activity in the dialyzed pellet and supernatant fractions obtained from *S. salivarius*, *S. sanguis* and various strains of *S. mutans* following sonic breakage and centrifugation at 30,000 *g*.

Organisms	30,000 *g* pellet	30,000 *g* supernatant
S. salivarius	1.00[a]	1.68
S. sanguis 10556	1.54	4.00
S. mutans AHT	1.68	4.31
S. mutans E-49	1.59	5.71
S. mutans PS-14	1.81	1.90
S. mutans OMZ-176	1.40	7.74
S. mutans 6715	1.40	3.00

[a] pmoles cyclic AMP bound/mg protein. Counts due to non-specific binding have been subtracted.

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References

[1] E.G. Krebs, in: Current topics in cellular regulation, Vol. 6, eds. B.L. Horecker and E.R. Stadtman (Academic Press, New York, 1972) p. 99.
[2] J.F. Kuo and P. Greengard, Proc. Natl. Acad. Sci. U.S. 64 (1969) 1349.
[3] D.A. Walsh, J.P. Perkins and E.G. Krebs, J. Biol. Chem. 243 (1968) 3763.
[4] M.A. Brostrom, E.M. Reimann, D.A. Walsh and E.G. Krebs, Advan. Enzyme Regul. 8 (1970) 191.
[5] L.J. Chen and D.A. Walsh, Biochemistry 10 (1971) 3614.
[6] T.A. Langan, in: Regulatory mechanisms for protein synthesis in mammalian cell, eds. A. San Pietro, M.R. Lamborg and F.T. Kenney (Academic Press, New York, 1968) p. 101.
[7] G.C. Majumdar and R.W. Turkinton, J. Biol. Chem. 246 (1971) 2650.

[8] H. Maeno, E.M. Johnson and P. Greengard, J. Biol. Chem. 246 (1971) 134.
[9] G.M. Walton, G.N. Gill, I.B. Abrass and L.D. Garren, Proc. Natl. Acad. Sci. U.S. 68 (1971) 880.
[10] J.F. Kuo and P. Greengard, J. Biol. Chem. 244 (1969) 3417.
[11] G.D. Kuehn, J. Biol. Chem. 246 (1971) 6366.
[12] R.L. Khandelwal and I.R. Hamilton, J. Biol. Chem. 246 (1971) 3297.
[13] R.L. Khandelwal and I.R. Hamilton, Arch. Biochem. Biophys. 151 (1972) 75.
[14] I.R. Hamilton, Can. J. Microbiol. 14 (1968) 65.
[15] E.M. Reimann, D.A. Walsh and E.G. Krebs, J. Biol. Chem. 246 (1971) 1986.
[16] D.A. Walsh, J.P. Perkins, C.O. Brostrom, E.S. Ho and E.G. Krebs, J. Biol. Chem. 246 (1971) 1968.
[17] W.B. Anderson, A.B. Schneider, M. Emmer, R.L. Perlman and I. Pastan, J. Biol. Chem. 246 (1971) 5929.
[18] M. Wilchek, Y. Salomon, M. Lowe and Z. Selinger, Biochem. Biophys. Res. Commun. 45 (1971) 1177.
[19] G.N. Gill and L.D. Garren, Biochem. Biophys. Res. Commun. 39 (1970) 335.