Proc. Natl. Acad. Sci. USA
Vol. 82, pp. 4578–4581, July 1985
Biochemistry

Inactivation of *S*-adenosyl-L-homocysteine hydrolase by cAMP results from dissociation of enzyme-bound NAD^+

(cellular differentiation/*Dictyostelium discoideum*/nucleotide cofactors/transmethylation/enzyme inactivation and reactivation)

R. J. HOHMAN*, M. C. GUITTON, AND M. VERON

Unité de Biochimie Cellulaire, Département de Biochimie et Génétique Moleculaire, Institut Pasteur, 28 rue du Dr. Roux, 75724 Paris Cedex 15, France

Communicated by E. R. Stadtman, March 6, 1985

ABSTRACT ***S*-Adenosyl-L-homocysteine hydrolase (EC 3.3.1.1) is inactivated by cAMP and also by 2′-deoxyadenosine, and in both cases, activity is restored by incubating the inactivated enzyme with NAD^+. We have previously presented evidence that, despite these similarities, inactivation by these two ligands proceeds by different mechanisms. We have now used a fluorescence technique to quantitate enzyme-bound NAD^+ and NADH on *S*-adenosyl-L-homocysteine hydrolase from *Dictyostelium discoideum*, and we have confirmed that cAMP and 2′-deoxyadenosine inactivate by different mechanisms. Whereas inactivation by 2′-deoxyadenosine is due to reduction of the enzyme-bound NAD^+ to NADH, incubation of *S*-adenosyl-L-homocysteine hydrolase with cAMP results in dissociation of the enzyme-bound NAD^+. The dissociation is reversible, and reactivation likely occurs by restoration of the initial NAD^+ content. This reversible inactivation by cAMP may be a mechanism of controlling biological methylation reactions by adjusting intracellular concentrations of *S*-adenosyl-L-homocysteine through action of *S*-adenosyl-L-homocysteine hydrolase.**

S-Adenosyl-L-homocysteine hydrolase (EC 3.3.1.1) is the enzyme present in eukaryotic cells that catalyzes the reversible cleavage of *S*-adenosyl-L-homocysteine (AdoHcy) into adenosine and homocysteine (1). AdoHcy is a product, and potent feedback inhibitor, of all *S*-adenosyl-L-methionine-dependent methylation reactions (2). Since the K_i for AdoHcy of various transmethylases varies over a 1000-fold range (3), it has been suggested that specific methylation reactions may be controlled by regulating intracellular concentrations of AdoHcy by means of AdoHcy hydrolase (4).

The enzyme is a tetramer (subunit M_r = 47,000). It contains tightly bound NAD^+, which is reduced to NADH and then reoxidized during the catalytic cycle (5). The inhibitory properties of a large number of substrate analogs have been studied (6–8) because of their potential use as antiviral or anticancer drugs. Furthermore, some of these inhibitors are being used to investigate the complicated catalytic and regulatory mechanisms of AdoHcy hydrolase. One adenosine analog, 2′-deoxyadenosine, has been extensively studied; it is a potent inhibitor of the enzyme. A mechanism of inactivation has been proposed (9) in which 2′-deoxyadenosine binds to the active site and an abortive catalytic cycle ensues that traps the AdoHcy hydrolase in an inactive enzyme-NADH configuration. Although 2′-deoxyadenosine has been described as an irreversible suicide inhibitor (9, 10), we have demonstrated that AdoHcy hydrolase inactivated by 2′-deoxyadenosine is reactivated by incubation with NAD^+ (11, 12).

The binding of cAMP to AdoHcy hydrolase has been known for some time (13, 14); however, there never was a function assigned to this ligand. To determine if cAMP regulates the activity of AdoHcy hydrolase, we purified and characterized this enzyme from *Dictyostelium discoideum* (12). We found that, in addition to the tightly bound NAD^+, AdoHcy hydrolase binds additional NAD^+ that both increases enzymatic activity and stabilizes subunit–subunit interaction. We also showed that the enzyme is inactivated upon incubation with cAMP. Although the enzyme remained inactive after the cAMP had been removed, enzymatic activity was completely restored by incubating the inactive enzyme with NAD^+.

As has been reported for the mammalian enzyme (9, 10), AdoHcy hydrolase from *D. discoideum* is also inactivated by 2′-deoxyadenosine (12). As we observed with cAMP-inactivated AdoHcy hydrolase, inactivation by 2′-deoxyadenosine is reversed by incubation with NAD^+. Despite these similarities, we argued that the mechanism of inactivation by these two ligands is likely to be different. cAMP lacks the 3′-OH group that is necessary for inactivation by 2′-deoxyadenosine. Moreover, whereas 2′-deoxyadenosine binds at the active site, cAMP likely has a separate binding site (6, 12).

Direct evidence for different mechanisms of inactivation can be obtained by analysis of enzyme-bound nucleotides. We have used a fluorescence technique to measure the amount of NAD^+ and NADH associated with both native AdoHcy hydrolase and enzyme inactivated with either cAMP or 2′-deoxyadenosine. Whereas 2′-deoxyadenosine inactivates by reducing enzyme-bound NAD^+, inactivation by cAMP results from dissociation of the enzyme-bound NAD^+. In both cases, the enzyme is reactivated by restoring the original NAD^+ content.

MATERIALS AND METHODS

Material. Unless otherwise noted, all chemicals were purchased from Sigma. The proteinase K, alcohol dehydrogenase, and lactate dehydrogenase were from Boehringer Mannheim.

Enzyme Purification and Activity Measurements. Procedures for culturing the cells and purifying the enzyme have been described (12). AdoHcy hydrolase was assayed in the synthesis direction at 25°C, using 10 μM [^{3}H]adenosine as reported (12). The enzyme concentration was calculated by using a specific activity for pure enzyme of 0.3 unit/mg. The incubation conditions for inactivation by the various ligands are described in the legends of the tables.

Quantitation of NAD^+ and NADH. (i) *Principle and sample preparation.* Both NAD^+ and NADH were measured by monitoring the intrinsic fluorescence emitted by NADH at 460 nm upon excitation at 340 nm. NADH was measured directly, whereas NAD^+ was first converted to NADH by alcohol dehydrogenase in the presence of ethanol. The

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Abbreviation: AdoHcy, *S*-adenosyl-L-homocysteine.

*Present address: Bldg. 10, Rm. 9N240, NIH, Bethesda, MD 20205.

fluorescent material was positively identified as NADH by following the fluorescence decrease as NADH was oxidized to NAD^+ by lactate dehydrogenase in the presence of pyruvate.

The enzyme-bound nucleotides are not substrates for lactate dehydrogenase or alcohol dehydrogenase. To release the nucleotides, 3 μl of proteinase K (8 mg/ml in 100 mM sodium phosphate, pH 7.0) was added to 120 μl of AdoHcy hydrolase, which was then incubated for 25 min at 30°C. The proteinase K was inactivated by incubating the samples at 100°C for 2 min. Precipitated material was removed by centrifugation and the supernatant solution was analyzed for NAD^+ and NADH. Control experiments with authentic NAD^+ and NADH demonstrated that these nucleotides were stable to this treatment.

(ii) *Fluorescence measurements.* Both NADH and NAD^+ were quantitated successively on the same sample. In a typical measurement, 800 μl of 12 mM sodium phosphate (pH 8.5) and 7 μl of ethanol were mixed together in a quartz cuvette (at 20°C) which was placed in a spectrofluorometer with excitation and emission monochrometers set to 340 nm and 460 nm, respectively. The sample of digested AdoHcy hydrolase, prepared as described above, was added and the increase in fluorescence was measured. After correction for nonspecific fluorescence (see below), this signal was compared with a standard curve and the concentration of NADH in the sample was calculated. Next, 2 μl of alcohol dehydrogenase was added and the increase in fluorescence due to reduction of NAD^+ to NADH was recorded. At this point, known quantities of NAD^+ were successively added and the corresponding fluorescence was recorded. This provided a calibration under conditions used for the actual measurement. This method enabled the detection of 0.1 nmol of NADH or NAD^+. All values reported are the average of triplicate measurements.

(iii) *Corrections for nonspecific fluorescence.* Control experiments suggested that not all of the material that fluoresces when AdoHcy hydrolase is initially added to the cuvette is β-NADH. Therefore, the data had to be corrected for this nonspecific fluorescence. For this purpose, a cuvette containing 800 μl of 10 mM sodium phosphate (pH 7.0) and 0.12 mM sodium pyruvate was placed in the spectrofluorometer and a baseline was recorded. An aliquot of proteolyzed AdoHcy hydrolase, prepared as described above, was added and the fluorescence signal was recorded. Next, 2 μl of lactate dehydrogenase (1:100 dilution, Boehringer Mannheim catalog no. 127-221) was added and the decrease in fluorescence due to oxidation of NADH to NAD^+ was measured. When the same experiment was performed with authentic NADH, greater than 97% of the fluorescence signal was eliminated upon addition of lactate dehydrogenase. With AdoHcy hydrolase, the fluorescence decreased only 75%. The remaining 25% was assumed to represent the non-NADH fluorescence. All NADH determinations were corrected accordingly.

Effect of cAMP, 2′-Deoxyadenosine, and 3′-Deoxyadenosine on the AdoHcy Hydrolase Assay. In this paper, we present results of inactivation of AdoHcy hydrolase by various ligands. To discriminate between inactivation of AdoHcy hydrolase and inhibition of the assay, we examined the effect of these ligands when they were added directly to the standard assay mixture (i.e., 10 μM [^{3}H]adenosine). As seen in Fig. 1, neither 400 μM cAMP nor 100 μM 2′-deoxyadenosine inhibited the assay. Even the most potent inhibitor, 3′-deoxyadenosine, produced only 5% inhibition at 50 μM. Since the carry-over of ligand from the inactivation medium to the assay was never greater than 0.5 μM, none of the inactivation reported here was due to inhibition of the AdoHcy hydrolase assay.

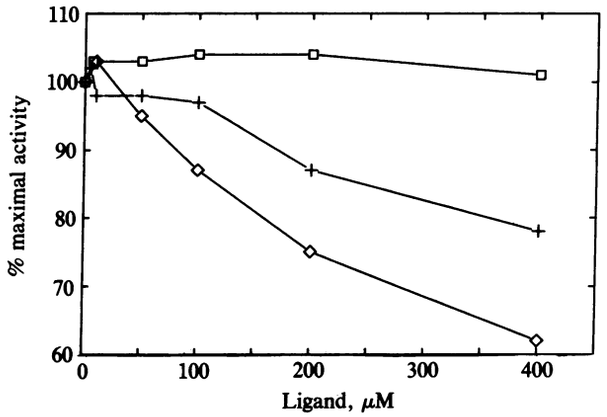

FIG. 1. Effect of cAMP, 2′-deoxyadenosine, and 3′-deoxyadenosine on the AdoHcy hydrolase assay. AdoHcy hydrolase was assayed under standard assay conditions in the presence of cAMP (□), 2′-deoxyadenosine (+), or 3′-deoxyadenosine (◇).

RESULTS

Nucleotide Content of Native AdoHcy Hydrolase. Freshly prepared AdoHcy hydrolase from *D. discoideum* was digested with proteinase K and the nucleotide content was determined. As seen in Table 1, this enzyme preparation contained equal amounts of NAD^+ and NADH (0.8–0.9 mol of each nucleotide per mol of tetramer). Although NAD^+ has been measured on native AdoHcy hydrolase from beef liver (5) and NADH has been found on enzyme inactivated with 2′-deoxyadenosine (9), NADH had not been reported on native enzyme.

NAD^+ and NADH were quantitated on several different preparations of AdoHcy hydrolase from *D. discoideum* by using the fluorescence technique. In each preparation, the native enzyme contained equal amounts of NAD^+ and NADH. Calculations of the stoichiometry yielded values of between two and four nucleotides per tetramer.

Nucleotide Content of Inactive Enzyme. To determine the difference in the mechanism of inactivation by cAMP and 2′-deoxyadenosine, AdoHcy hydrolase was inactivated by one of these ligands under the conditions described in Table 1. As mentioned above, the control (no additions) contained equal amounts of NAD^+ and NADH. The AdoHcy hydrolase that was incubated with 2′-deoxyadenosine was inactivated with a parallel reduction of enzyme-bound NAD^+ to NADH. This is the same mechanism as has been reported for the bovine AdoHcy hydrolase (9).

AdoHcy hydrolase incubated with cAMP was inactivated 75% (3.5 hr) or 77% (17 hr). However, unlike inactivation by 2′-deoxyadenosine, no reduction of NAD^+ to NADH occurred. These results confirm our earlier proposal that inactivation by cAMP and inactivation by 2′-deoxyadenosine proceed by different mechanisms.

Since NAD^+ reactivates both cAMP-inactivated and 2′-deoxyadenosine-inactivated AdoHcy hydrolase (see below), it seemed likely that inactivation by cAMP also involves the enzyme-bound NAD^+. One possibility is that incubation of the enzyme with cAMP results in dissociation of NAD^+, which we would not have observed due to the design of the experiment. The inactivation and all subsequent sample preparation for nucleotide determination for the experiments described in Table 1 were performed in the same tube to avoid losses that might occur when transferring small volumes. Therefore, those experiments would not distinguish between nucleotide released from AdoHcy hydrolase during inactivation and that released during protease treatment.

To determine if NAD^+ is released from the enzyme during inactivation, AdoHcy hydrolase was incubated with either

Table 1. Nucleotide contents of native and inactivated AdoHcy hydrolase

Inactivating ligand	Exp.	% inactivation	Nucleotide content, mol/mol of tetramer			% reduction
			NAD^+	NADH	Total	$NAD^+ \rightarrow$ NADH*
None	1	0	0.86	0.86	1.72	0
	2	0	0.81	0.88	1.69	0
2'-Deoxyadenosine	1	45	0.55	1.17	1.72	36
	2	71	0.16	1.56	1.72	80
cAMP	1	75	0.86	0.86	1.72	0
	2	77	0.88	0.81	1.69	0

AdoHcy hydrolase (0.29 unit/ml) was incubated for either 3.5 hr (experiment 1) or 13 hr (experiment 2) in buffer containing 60 mM sodium phosphate (pH 6.5), 50 mM NaCl, 2 mM EDTA, and either 100 μM 2'-deoxyadenosine or 100 μM cAMP. The control was incubated at 4°C and contained no additions. After incubation, residual AdoHcy hydrolase activity was measured and the samples were prepared and analyzed for nucleotide content. All values are the average of three or more measurements.
*The amount of NAD^+ that was reduced to NADH during inactivation was calculated on a given sample from the difference in NAD^+ content before and after inactivation.

cAMP or 2'-deoxyadenosine as described in the legend to Table 1. Instead of being digested with protease at the end of incubation, the enzyme solution was filtered through a Centrifree Micropartition System (Amicon) to separate protein from unbound ligands, and the supernatants were analyzed for NAD^+ and NADH. The results are shown in Table 2. No ligand was released from either the control enzyme or the AdoHcy hydrolase inactivated with 2'-deoxyadenosine. However, the AdoHcy hydrolase that was inactivated 77% by cAMP released 73% of its enzyme-bound NAD^+. This represents NAD^+ that dissociated from the enzyme during incubation with cAMP, since no NAD^+ was found in the ultrafiltrate of the control enzyme or AdoHcy hydrolase inactivated with 2'-deoxyadenosine.

Kinetics of cAMP-Dependent Inactivation of AdoHcy Hydrolase vs. Enzyme Concentration. We previously reported that the activity of AdoHcy hydrolase inactivated by cAMP was completely restored by further incubation with NAD^+, even in the presence of cAMP (11). Moreover, as seen in Table 1, inactivation at high concentrations of AdoHcy hydrolase (5 μM tetramer) reaches a plateau and 25% of the initial activity remains even after prolonged incubation. Considering the cAMP-induced dissociation of NAD^+ demonstrated above, these results suggest that an equilibrium is established between dissociation of NAD^+ from the enzyme and reassociation of the dissociated NAD^+. If this is true, the percent inactivation at equilibrium will be reduced at high AdoHcy hydrolase concentrations since the absolute concentration of dissociated NAD^+ will be greater.

Table 2. Dissociation of NAD^+ upon inactivation of AdoHcy hydrolase by cAMP

Inactivating ligand	% inactivation	% of total nucleotide released from the enzyme	
		NAD^+	NADH
None	0	0	0
2'-Deoxyadenosine	71	0	0
cAMP	77	73	0

AdoHcy hydrolase was incubated for 13 hr under the same conditions described for experiment 2 in Table 1. Instead of being treated with proteinase K, the enzyme solution was filtered through a Centrifree Micropartition System (Amicon) to separate protein from unbound ligands. The nucleotide present in the ultrafiltrate corresponds to ligand released from the enzyme during incubation with cAMP. The ultrafiltrates were checked to confirm that there was no protein present. All values are corrected for a small amount of fluorescent material leached from the filters.

To test this hypothesis, two different concentrations of AdoHcy hydrolase (0.16 and 3.7 μM) were incubated with two different concentrations of cAMP (30 and 100 μM) and the kinetics of inactivation were followed. As seen in Fig. 2, the rate of inactivation during the first 4 hr was a function of cAMP concentration and was independent of enzyme concentration. In the two tubes containing 0.16 μM AdoHcy hydrolase, inactivation continued until the enzyme was completely inactive. However, in the two tubes containing 3.7 μM AdoHcy hydrolase, inactivation reached a plateau after 4 hr (with 30 μM cAMP) or 8 hr (with 100 μM cAMP). Therefore, we conclude that NAD^+ dissociated from AdoHcy hydrolase during inactivation by cAMP is capable of reassociating with the enzyme when the concentration of dissociated NAD^+ reaches the micromolar range.

DISCUSSION

The data presented here establish a second mechanism for inactivating AdoHcy hydrolase. Incubating the enzyme with cAMP results in dissociation of the enzyme-bound NAD^+, and restoring the initial NAD^+ content reactivates the enzyme.

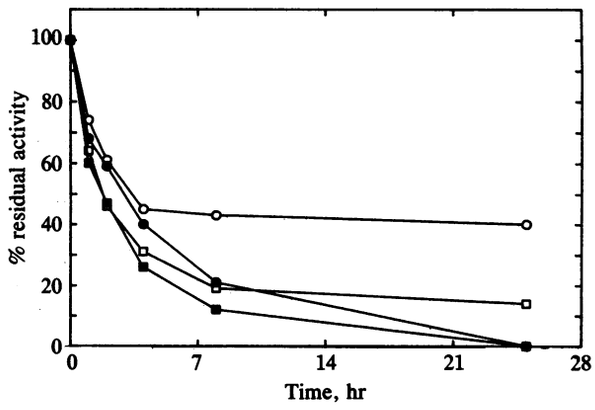

FIG. 2. Kinetics of inactivation by cAMP at two different concentrations of AdoHcy hydrolase. AdoHcy hydrolase at 0.16 μM (filled symbols) or 3.7 μM (open symbols) was incubated with either 30 μM cAMP (circles) or 100 μM cAMP (squares) at 25°C in buffer containing 20 mM sodium phosphate (pH 6.5) and 20 mM NaCl. At the times indicated, aliquots were removed and assayed. Controls were incubated at 4°C in the absence of cAMP and remained fully active throughout the course of the experiment. All values reported are the average of three or more measurements.

Inactivation of AdoHcy hydrolase by cAMP is a relatively slow process, with a half-life of 1.5 hr at 25°C in the presence of 100 μM cAMP (Fig. 2). At any time, activity can be completely restored by adding back NAD^+ (11, 12). Moreover, the data in Fig. 2 suggest that dissociated NAD^+ is able to reassociate. Inactivation by cAMP at the higher concentration of AdoHcy hydrolase is incomplete, and the extent of inactivation is greater at the higher concentration of cAMP. Meanwhile, inactivation at the lower AdoHcy hydrolase concentration is complete. From these results, we conclude that inactivation of 3.7 μM AdoHcy hydrolase stops when an equilibrium is established between the dissociation and reassociation of NAD^+. Inactivation at 0.16 μM AdoHcy hydrolase proceeds to completion because the concentration of dissociated NAD^+ is too low for reassociation to occur.

The magnitude of cAMP-dependent inactivation is proportional to the decrease in enzyme-bound NAD^+, but the same relationship does not hold for the inactivation produced by 2'-deoxyadenosine. The data in Table 1 show that AdoHcy hydrolase was 92% inactivated when 60% of its NAD^+ was reduced to NADH. A similar phenomenon was reported with the beef liver enzyme (9), which was completely inactivated when 50% of the enzyme-bound NAD^+ was reduced to NADH. Therefore, enzyme-bound NAD^+ must have a function (or functions) other than its role in catalysis. Since adding NAD^+ to freshly prepared AdoHcy hydrolase both increases catalytic activity and stabilizes subunit–subunit interactions (12), it is likely that NAD^+ also plays a structural role. This hypothesis is supported by our previous observation that inactivated AdoHcy hydrolase that has been dialyzed and stored in the absence of nucleotide is unstable and loses its ability to be reactivated (12).

NADH has been found on AdoHcy hydrolase from beef liver only after inactivation by 2'-deoxyadenosine (9). In contrast, we find NADH associated with freshly prepared AdoHcy hydrolase from *D. discoideum*. The function of the enzyme-bound NADH remains unclear. The simplest explanation is that this NADH is the result of *in vivo* inactivation involving reduction of NAD^+. Alternatively, this NADH is not related to the nucleotide involved in catalysis and may have its own binding site. The latter hypothesis is supported by data showing that not all of the enzyme-bound nucleotide participates in catalysis (9) and also that addition of NAD^+ (or NADH) stabilizes the quaternary structure during electrophoresis (12). Furthermore, this could explain why NAD^+, but not NADH, is released from the enzyme during inactivation by cAMP (see Table 2).

The most intriguing question raised by our findings is whether inactivation by cAMP plays a physiological role. At this time, the only generally accepted role of cAMP in regulating enzymatic activity is the activation of cAMP-dependent protein kinases (15). However, the importance of biological methylation reactions as well as the selective sensitivity of various transmethylases to AdoHcy has been well established (4, 16). Moreover, cAMP is essential to the differentiation of *D. discoideum* both as an intracellular messenger during terminal differentiation (17) and also for transcription and stability of a class of developmentally regulated mRNAs (18–21). We speculate that one of the functions of cAMP is to control a vital transmethylase by regulating the intracellular concentration of AdoHcy by means of AdoHcy hydrolase.

We thank Dr. Georges N. Cohen for his continued interest and helpful discussions. We also thank Drs. M. C. Rebiere, R. L. Levine, and H. Metzger for critical comments on the manuscript. This work was supported by a grant from the Centre National de la Recherche Scientifique (Action Thématique Programmée no. 955113); R.J.H. was a recipient of fellowships from the Foundation Philippe and the Foundation Pour la Recherche Médicale.

1. de la Haba, G. & Cantoni, G. L. (1959) *J. Biol. Chem.* **234,** 603–608.
2. Mann, J. D. & Mudol, S. H. (1963) *J. Biol. Chem.* **238,** 381–385.
3. Kredich, N. M. & Hershfield, M. S. (1980) *Adv. Enzyme Regul.* **18,** 181–191.
4. Usdin, E., Borchardt, R. T. & Creveling, E. R., eds. (1979) *Transmethylation* (Elsevier/North-Holland, New York).
5. Palmer, J. L. & Abeles, R. H. (1979) *J. Biol. Chem.* **254,** 1217–1226.
6. Chiang, P. K., Guranowski, A. & Segall, J. E. (1981) *Arch. Biochem. Biophys.* **207,** 175–184.
7. Hershfield, M. S., Kredich, N. M., Small, W. C. & Fredericksen, M. L. (1979) in *Transmethylation*, eds. Usdin, E., Borchardt, R. T. & Creveling, C. R. (Elsevier/North-Holland, New York), pp. 173–180.
8. Pugh, C. S. G. & Borchardt, R. T. (1982) *Biochemistry* **21,** 1535–1541.
9. Abeles, R. H., Fish, S. & Lipinskas, B. (1982) *Biochemistry* **21,** 5557–5562.
10. Hershfield, M. S. (1979) *J. Biol. Chem.* **254,** 22–25.
11. Hohman, R. J. & Veron, M. (1984) *FEBS Lett.* **165,** 265–268.
12. Hohman, R. J., Guitton, M. C. & Veron, M. (1984) *Arch. Biochem. Biophys.* **233,** 785–795.
13. Hershfield, M. S. & Kredich, N. M. (1978) *Science* **202,** 757–760.
14. Saebo, J. & Ueland, P. M. C. (1978) *FEBS Lett.* **96,** 125–128.
15. Schramm, M. & Selinger, Z. (1984) *Science* **225,** 1350–1356.
16. Usdin, E., Borchardt, R. T. & Creveling, C. R., eds. (1982) *Biochemistry of S-Adenosylmethionine and Related Compounds* (Macmillan, London).
17. Kay, R. R. (1982) *Proc. Natl. Acad. Sci. USA* **79,** 3228–3231.
18. Williams, J. G., Tsang, A. S. & Mahbubani, H. (1980) *Proc. Natl. Acad. Sci. USA* **77,** 7171–7175.
19. Loomis, W. F., ed. (1982) *The Development of Dictyostelium discoideum* (Academic, New York).
20. Mangiarotti, G., Ceccarelli, A. & Lodish, H. F. (1983) *Nature (London)* **301,** 616–618.
21. Medhy, M. C., Ratner, D. & Firtel, R. A. (1983) *Cell* **32,** 763–771.