BBA 65670

REGULATION OF AMP DEAMINASE BY 2,3-DIPHOSPHOGLYCERIC ACID: A POSSIBLE MECHANISM FOR THE CONTROL OF ADENINE NUCLEOTIDE METABOLISM IN HUMAN ERYTHROCYTES

A. ASKARI AND S. N. RAO
Department of Pharmacology, Cornell University Medical College, New York, N.Y. (U.S.A.)

(Received July 13th, 1967)

SUMMARY

1. AMP deaminase (AMP aminohydrolase, EC 3.5.4.6) activities of dialyzed and undialyzed hemolyzates of human erythrocytes in the presence of optimal concentrations of K^+ were measured. Whereas the activity of the dialyzed hemolyzate was a linear function of the hemolyzate concentration, that of the undialyzed hemolyzate was not. These results suggested the presence of a dialyzable inhibitor of the enzyme in the hemolyzate.

2. Effects of several organic phosphates of erythrocytes on AMP deaminase were studied. 2,3-Diphosphoglyceric acid, at concentrations usually found in erythrocytes, was found to be an inhibitor of the enzyme.

3. The substrate-velocity curve of AMP deaminase is a sigmoid. In the presence of 2,3-diphosphoglyceric acid the curve became more sigmoidal in shape. The effects of varying concentrations of 2,3-diphosphoglyceric acid on AMP deaminase in the presence and absence of ATP were also determined. In the presence of ATP, when the per cent inhibition of velocity was plotted against the concentration of 2,3-diphosphoglyceric acid a sigmoidal curve was obtained.

4. The data suggest that 2,3-diphosphoglyceric acid may control the adenine nucleotide content of the intact erythrocyte through an allosteric regulation of the AMP deaminase activity.

INTRODUCTION

The presence of high levels of AMP deaminase (AMP aminohydrolase, EC 3.5.4.6) activity in human erythrocytes has been known for a long time[1,2]. It has also been established that the mature human erythrocyte does not have the ability either to synthesize the purine portion of adenine nucleotides from purine ring precursors, or to convert IMP to AMP[3,4]. These facts have led to the realization that the control of AMP deaminase activity in the intact cell may be crucial to the preservation of the adenine nucleotide content of the erythrocyte[5,6]. CONWAY AND COOKE[1], in an early

report, suggested that AMP deaminase was present in a partially inactive form within the intact cell. Recently, in the course of investigating the inhibitory effects of inorganic phosphate on AMP deaminase, we suggested the possible role of this anion in the control of AMP deaminase activity[6]. However, considering the relatively high levels of ATP (a modifier of AMP deaminase which can counteract the P_i inhibition[6]) in the intact cell, it did not seem that the normal cellular concentration of P_i could exert sufficient inhibitory effect on the enzyme. In search of other possible cellular modifiers of AMP deaminase activity, we have now found that 2,3-diphosphoglyceric acid, at concentrations usually found in the red cell, is an inhibitor of the enzyme. In this report we present these findings, the results of studies on the kinetics of 2,3-diphosphoglyceric acid effect on the enzyme, and a discussion of the possible physiological significance of this effect of 2,3-diphosphoglyceric acid.

MATERIALS AND METHODS

Nucleotides were obtained from Sigma Chemical Co., St. Louis, Mo. All other organic phosphates, as either the sodium salts or the cyclohexylammonium salts, were purchased from Boehringer Mannheim Corp., New York, N.Y. and Calbiochem, Los Angeles, Calif. The organic phosphates, as well as the other common Reagent Grade chemicals, were used without further purification.

Methods for the preparation of enzyme source and the assay of enzyme activity have been described[7]. P_i was determined according to FISKE AND SUBBAROW[8].

RESULTS

CONWAY AND COOKE[1] found that the AMP deaminase activity of the hemolyzate of human red cells was not a linear function of the hemolyzate concentration. On this basis, they suggested the binding of the enzyme to an inhibitor in the intact cells and cell hemolyzates. When these experiments were done, the activating effects of alkali cations on AMP deaminase[7,9,10] were not known; and from the reported experimental conditions the possibility of the presence of different levels of activator cations in the various assay solutions can not be ruled out. Therefore, we performed similar experiments measuring the AMP deaminase activities of various amounts of dialyzed and undialyzed hemolyzates in the presence of optimal concentrations of K^+. The results, presented in Fig. 1, show that whereas the activity of the dialyzed hemolyzate is a linear function of the hemolyzate concentration, that of the undialyzed hemolyzate is not. These data strongly suggest the presence of a dialyzable inhibitor in the hemolyzate of fresh red cells. Table I shows the results of phosphate determinations on two of the reaction mixtures of Fig. 1 containing the highest concentrations of dialyzed and undialyzed hemolyzates. Also included in Table I are calculations showing the expected inhibitory effect of the measured phosphate of the undialyzed hemolyzate, and the actual inhibition of the enzyme activity in the undialyzed hemolyzate. Comparison of the data clearly shows that the major portion of the inhibition of AMP deaminase activity in the undialyzed hemolyzate can not be ascribed to P_i. Since previous work of our laboratory[6] had already established that some simple anions are capable of modifying the activity of AMP deaminase, we were prompted to study the possible modifying effects of the cellular organic phosphates on the enzyme activity.

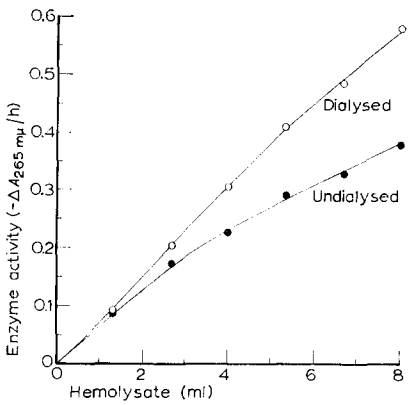


Fig. 1. Comparison of AMP deaminase activities of dialyzed and undialyzed hemolyzates of human erythrocytes. Cells from freshly drawn citrated blood were obtained by centrifugation and washed in saline. Packed cells were hemolyzed in 5 vol. of water and centrifuged at 20 000 × *g* for 0.5 h. The supernatant solution was carefully separated from the ghosts. A portion of this solution was dialyzed against 100 vol. of water for 24 h at 4°. The rest was kept for 24 h at 4° without dialysis. The indicated aliquots of the dialyzed and undialyzed hemolyzates were incubated at 37° with 20 μmoles of AMP, 0.8 mmole of Tris–HCl (pH 7.2), and 1 mmole of KCl in a final volume of 10 ml. Sets of controls, without the hemolyzate or without AMP, were also included. After preliminary experiments, appropriate incubation times were chosen so that the measured activities were initial velocities[6]. Each reaction mixture was deproteinized by the addition of 6 ml of 8% $HClO_4$. Absorbances at 265 mμ of 1 to 25 dilutions of the deproteinized solutions were measured.

The effects of normal nucleotides of the red cell on the enzyme activity have already been reported[7]. In Table II the effects of a variety of organic phosphates, not tested before, are shown. The previously described effects of ATP are also included in the table for the purpose of comparison. The results show that under the experimental conditions only 2,3-diphosphoglyceric acid has an inhibitory effect on the enzyme. To further characterize the effects of 2,3-diphosphoglyceric acid, some kinetic studies were attempted. In Fig. 2, the substrate–velocity curves in the absence of added

TABLE I

COMPARISONS OF AMP DEAMINASE ACTIVITIES, PHOSPHATE CONTENTS, AND CALCULATED INHIBITORY EFFECTS OF PHOSPHATE, OF DIALYZED AND UNDIALYZED HEMOLYZATES OF HUMAN ERYTHROCYTES

Aliquots of deproteinized solutions from the two reaction mixtures of Fig. 1 containing the highest amounts of dialyzed and undialyzed hemolyzates were assayed for P_i. Enzyme activities are the same as those shown in Fig. 1. The difference between the two enzyme activities, as per cent of the activity of the dialyzed hemolyzate, is expressed as per cent inhibition due to dialyzable inhibitor. Per cent inhibition due to P_i is calculated from previous data[6].

Hemolyzate	*Enzyme activity* ($-\Delta A_{265\ m\mu}$/*h*)	*Inhibition due to dialyzable inhibitor (%)*	*P_i content (mM)*	*Inhibition due to P_i (%)*
Dialyzed	0.55	—	0	0
Undialyzed	0.37	32	0.05	3 (approx.)

TABLE II

EFFECTS OF SOME ORGANIC PHOSPHATES ON AMP DEAMINASE OF HUMAN ERYTHROCYTES

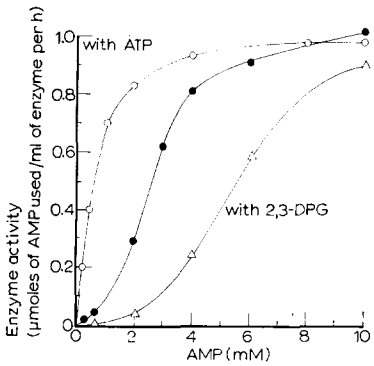
Each assay system contained 10 μmoles of AMP, 100 μmoles of Tris–HCl (pH 7.2), a fixed amount of enzyme solution, and the indicated amounts of KCl, NaCl, and the various phosphates. Final volume was 2.5 ml. The remainder of the assay procedure was as described before[7]. Enzyme activity in the presence of K^+, and the absence of any of the phosphates, was 0.41 ($-\Delta A_{265\ m\mu}$/ml of enzyme per h). The arbitrary value of 100 is assigned to this activity and all other activities are expressed relative to it.

Added phosphates (2 mM)	*Enzyme activity in the presence of*	
	Na^+ (100 mM)	*K^+ (100 mM)*
None	8	100
ATP	88	110
Glucose 1-phosphate	8	100
Glucose 6-phosphate	9	98
Fructose 6-phosphate	8	96
Fructose 1,6-diphosphate	7	99
Ribose 5-phosphate	10	102
2-Phosphoglyceric acid	11	97
3-Phosphoglyceric acid	8	96
2,3-Diphosphoglyceric acid*	2	1
Phosphoenolpyruvic acid	10	100

* Since the cyclohexylammonium salt of 2,3-diphosphoglyceric acid was used, in separate experiments it was shown that cyclohexylammonium ion had no inhibitory effect on the enzyme activity.

modifier, in the presence of 2,3-diphosphoglyceric acid, and in the presence of ATP, are shown. In agreement with our previous results[6], and the results of SETLOW AND LOWENSTEIN[11] on the deaminase of brain, the curve obtained in the presence of ATP is hyperbolic, and that obtained in the absence of added modifier is sigmoid. It is also evident that the presence of 2,3-diphosphoglyceric acid does not affect the maximum velocity, but that it shifts the sigmoid substrate–velocity curve to the right. It is now well-recognized[12] that kinetic results of the type obtained with AMP deaminase are not necessarily indicative of the presence of subunit structure of the enzyme, and that a variety of mechanisms, some involving cooperative interactions, are consistent with the kinetics. In the case of the erythrocyte AMP deaminase we have already pointed out[6] the possibility of the presence of at least two regulatory sites: one for the binding of activator cation and the other for the binding of the modifier anion. In this context, and considering the anionic nature of the substrate, it is difficult to decide whether the inhibitory effect of an anion such as 2,3-diphosphoglyceric acid is due to the interaction with the active site, or with the regulatory site, or with both. Regardless of the precise mechanism, however, the shift of the sigmoid substrate–velocity curve resulting from the presence of 2,3-diphosphoglyceric acid shows that the compound could be an efficient inhibitory regulator of AMP deaminase activity. The significance of such sigmoid kinetic curves in the control of cellular enzyme activities has been amply discussed[12,13].

In Fig. 3 the effects of varying concentrations of 2,3-diphosphoglyceric acid on the enzyme activity, in the presence and absence of a fixed concentration of ATP, are shown. It is evident that the inhibitory concentrations of 2,3-diphosphoglyceric acid



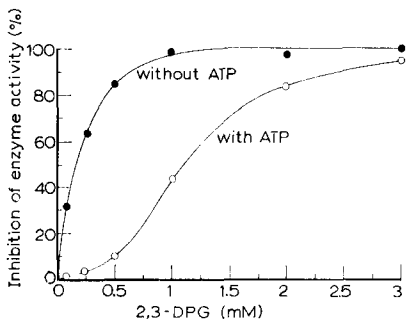


Fig. 2. Effect of varying substrate concentration on AMP deaminase activity of human erythrocytes. ●, in the absence of added modifier; ○, in the presence of 2 mM ATP; △, in the presence of 1 mM 2,3-diphosphoglyceric acid (2,3-DPG). In each reaction the concentration of K^+ was 100 mM. Other conditions were the same as described for Table II.

Fig. 3. Effect of varying 2,3-diphosphoglyceric acid (2,3-DPG) concentration on AMP deaminase activity of human erythrocytes in the absence and presence of 1 mM ATP. K^+ concentration was 100 mM in all reactions. Other conditions were the same as described for Table II.

are well within the normal cellular levels of the compound. Of special interest again is the pronounced sigmoid shape of the inhibition curve in the presence of ATP. These results indicate that in a situation where the enzyme is functioning in the presence of both ATP and 2,3-diphosphoglyceric acid (*e.g.* in the intact cell), a small change in the 2,3-diphosphoglyceric acid concentration within a narrow range, could cause the conversion of a highly active enzyme to a highly inactive enzyme, or *vice versa*.

DISCUSSION

The mature non-nucleated human red cell contains large quantities of 2,3-diphosphoglyceric acid[14]. Although the role of this compound in the enzymic conversion of 3-phosphoglyceric acid to 2-phosphoglyceric acid has been established[15], the physiological significance of the large quantities in the red cell, as compared to those in other tissues, has not been apparent. The suggestion that a pool of 2,3-diphosphoglyceric acid may be important to the red cell economy as a ready source of P_i has been made[16]. Regulatory roles of 2,3-diphosphoglyceric acid on glycolysis and pentose phosphate metabolism have also been suggested[17] on the basis of observations on the inhibitory effects of the compound on transketolase, transaldolase, and several transphosphorylases of the glycolytic pathway. The recent work of BENESCH AND BENESCH[18] indicates that 2,3-diphosphoglyceric acid can be a regulator of the allosteric properties of the red cell hemoglobin. The results presented in this paper suggest another possible physiological role of 2,3-diphosphoglyceric acid. As pointed out in INTRODUCTION, the available evidence indicates that the enzymic conversion of AMP to IMP represents an irreversible loss of the adenine nucleotide content of the red cell. Our experiments show the presence of a dialyzable inhibitor of the AMP deaminase in the hemolyzates of fresh red cells. We have further shown that 2,3-diphosphoglyceric acid, at physiological concentrations, is an allosteric inhibitor of the enzyme. Although we did not attempt to demonstrate the presence of 2,3-diphosphoglyceric acid in the

hemolyzate, simple calculations based on the known normal levels of 2,3-diphosphoglyceric acid[19], show that the undialyzed hemolyzate as prepared by us should contain sufficient 2,3-diphosphoglyceric acid to account for all the inhibitory activity present in the hemolyzate. Therefore, it is not unreasonable to assume that under physiological conditions, 2,3-diphosphoglyceric acid is the agent which prevents or regulates the loss of adenine nucleotides of the red cell. The proof of this assumption must of course await the demonstration of a correlation between the 2,3-diphosphoglyceric levels and AMP deamination in the intact cell. It is noteworthy, however, that several experiments reported in the literature are consistent with such a hypothesis. The works of PRANKERD[20], BARTLETT AND BARNET[21], and GOMPERTS[22], on the chemical changes in red cells during cold storage or incubation show that the rate of decrease of cellular 2,3-diphosphoglyceric acid is greater than the rate of disappearance of adenine nucleotides. Furthermore, the data of BARTLETT AND BARNET[21] indicate a slow disappearance of adenine nucleotides at the early phases of storage, and a more rapid loss at the later stages. Interestingly, the beginning of the rapid phase of nucleotide loss coincides with the time at which most of the 2,3-diphosphoglyceric acid has already disappeared. These data, when considered together with our results, tend to support our suggestion on the regulatory role of 2,3-diphosphoglyceric acid in the adenine nucleotide metabolism of the red cell.

ACKNOWLEDGEMENTS

This investigation was supported by U.S. Public Health Service Research Grant No. HE 10884 from the National Heart Institute.

We thank Mr. L. HARA for his technical assistance in the performance of portions of this work.

REFERENCES

1 E. J. CONWAY AND R. COOKE, *Nature*, 142 (1938) 720.
2 E. J. CONWAY AND R. COOKE, *Biochem. J.*, 33 (1939) 479.
3 C. BISHOP, *J. Biol. Chem.*, 235 (1960) 3228.
4 B. A. LOWY, M. K. WILLIAMS AND I. M. LONDON, *J. Biol. Chem.*, 237 (1962) 1622.
5 C. BISHOP, in C. BISHOP AND D. M. SURGENOR, *The Red Blood Cell*, Academic Press, New York, 1964, p. 170.
6 A. ASKARI, *Mol. Pharmacol.*, 2 (1966) 518.
7 A. ASKARI AND J. E. FRANKLIN, Jr., *Biochim. Biophys. Acta*, 110 (1965) 162.
8 C. H. FISKE AND Y. SUBBAROW, *J. Biol. Chem.*, 66 (1925) 375.
9 A. ASKARI, *Science*, 141 (1963) 44.
10 A. ASKARI, *Nature*, 202 (1964) 185.
11 B. SETLOW AND J. M. LOWENSTEIN, *J. Biol. Chem.*, 242 (1967) 607.
12 E. R. STADTMAN, in F. F. NORD, *Advances in Enzymology*, Vol. 28, Interscience, New York, 1966, p. 41.
13 J. P. CHANGEUX, *Cold Spring Harbor Symp. Quant. Biol.*, 26 (1963) 497.
14 I. GREENWALD, *J. Biol. Chem.*, 63 (1925) 339.
15 E. W. SUTHERLAND, T. Z. POSTERNACK AND C. F. CORI, *J. Biol. Chem.*, 181 (1949) 155.
16 J. A. J. PRANKERD, *The Red Cell*, Thomas, Springfield, 1961, p. 63.
17 Z. DISCHE, in C. BISHOP AND D. M. SURGENOR, *The Red Blood Cell*, Academic Press, New York, 1964, p. 208.
18 R. BENESCH AND R. E. BENESCH, *Biochem. Biophys. Res. Commun.*, 26 (1967) 162.
19 G. R. BARTLETT, *J. Biol. Chem.*, 234 (1959) 449.
20 T. A. J. PRANKERD, *Biochem. J.*, 64 (1956) 209.
21 G. R. BARTLETT AND H. N. BARNET, *J. Clin. Invest.*, 39 (1960) 56.
22 B. D. GOMPERTS, *Biochem. J.*, 102 (1967) 782.