Carboxyl methylation of human erythrocyte band 3 in intact cells

Relation to anion transport activity

Lillian L. LOU* and Steven CLARKE†

Department of Chemistry and Biochemistry and the Molecular Biology Institute, University of California, Los Angeles, CA 90024, U.S.A.

The anion transport protein of the human erythrocyte membrane, band 3, is reversibly methylated by an endogenous protein carboxyl methyltransferase. The physiological consequence of this modification was studied by measuring the rate of phosphate transport by intact erythrocytes incubated under conditions where protein methylation reactions are inhibited. No change in phosphate transport was detected when cells were treated with either methionine-free media or cycloleucine, whereas cells incubated with adenosine and homocysteine thiolactone displayed a marginally slower rate of transport, which was not reversed by subsequent remethylation of the membrane proteins. These results suggest that erythrocyte protein carboxyl methylation does not directly regulate this activity of band 3.

INTRODUCTION

Protein carboxyl methyltransferases (*S*-adenosyl-L-methionine: protein *O*-methyltransferases) are widely distributed in nature and catalyse the formation of methyl esters on protein carboxylic acid residues [1]. For example, a specific bacterial methyltransferase is responsible for the methylation of several membrane polypeptides on L-glutamyl residues, and these reactions appear to regulate the sensory output of these chemoreceptor proteins [2–5]. Protein carboxyl methyltransferases are also present in many mammalian tissues. The physiological roles of these eukaryotic enzymes are poorly understood, although it has been hypothesized that these reactions can also regulate the function of the various methyl-accepting substrates [1, 5–7].

We have studied eukaryotic protein carboxyl methylation in human erythrocytes (for a review, see [1]). Several major methyl acceptors have been identified in the membrane, including the cytoskeletal proteins band 2.1 and band 4.1, as well as band 3, which catalyses anion transport across the erythrocyte membrane in the major physiological reaction of chloride and bicarbonate exchange [8–11]. The band 3 protein has been shown to incorporate base-labile methyl groups when intact cells were incubated with methionine [12], when lysed cells were incubated with AdoMet [13], and when isolated erythrocyte membranes were incubated with purified erythrocyte carboxyl methyltransferase and AdoMet [14, 15]. Based on these results, the possibility that erythrocyte anion transport is regulated by the reversible methylation of carboxyl groups on band 3 has been proposed [12, 15].

In the present study, we have investigated the physiological effects of the inhibition of protein carboxyl methylation reactions on the anion transport activity of band 3 in intact cells. The experiments reported here provide a direct test for a regulatory role of erythrocyte protein carboxyl methyltransferase on the activity of band 3 protein.

EXPERIMENTAL PROCEDURES

Preparation of erythrocytes and incubation with methylation inhibitors

Freshly drawn human erythrocytes were washed in 10 vol. of iso-osmotic phosphate (118 mM-sodium phosphate, pH 7.4) three times and were then incubated with or without inhibitors of methylation for 24 h as described [16]. Control-methylated samples contained cells at a 10% haematocrit incubated at 37 °C with vigorous shaking in iso-osmotic phosphate containing 0.1 mg/ml each of penicillin G and streptomycin sulphate, 16.7 mM-D-glucose and 0.1 mM-L-methionine (standard incubation buffer). Methylation-inhibited samples were incubated in standard incubation buffer, plus 50 mM-cycloleucine; or plus 5 mM each of adenosine and L-homocysteine thiolactone; or without added methionine. At the end of the incubation period, cells were collected by centrifugation and washed in 10 vol. of ice-cold iso-osmotic phosphate.

Phosphate transport assay

Phosphate uptake into erythrocytes was measured by the amount of radioactivity retained by cells incubated with [³²P]P_i. The procedures described in Ho & Guidotti [9] were employed with modifications. Packed cells were suspended in 0.974 vol. of sodium citrate buffer (0.109 M in sodium), pH 6.4. Cells were incubated for 15 min at the desired temperature in the buffer prior to assay. Assays were performed at 24 °C or 37 °C.

The reaction was initiated by addition of 0.026 vol. of 1 M-sodium phosphate, pH 6.4 containing [³²P]P_i, 1×10^5 – 4×10^5 c.p.m.). The final phosphate concentration in the assay buffer was 26 mM. The amounts of sodium phosphate and sodium citrate present were designed to achieve an osmolarity of 0.31 osm during the assay. Samples were vortex-mixed immediately and shaken continuously at the desired temperature. The reaction was quenched at defined time intervals by transferring

Abbreviations used: DIDS, 4,4'-di-isothiocyanato-2,2'-stilbene disulphonic acid; AdoHcy, *S*-adenosylhomocysteine; AdoMet, *S*-adenosylmethionine.

* Present address: Department of Pharmacology, Stanford University, Stanford, CA 94305, U.S.A.

† To whom correspondence and reprint requests should be addressed.

aliquots (0.13 ml) rapidly into Microfuge tubes containing 0.52 ml of an ice-cold solution of 12.5 μM -DIDS (a covalent inhibitor of anion transport [8, 17]) in a mixture of sodium citrate and sodium phosphate, pH 6.4 (0.31 osm). For the zero time points, aliquots were withdrawn immediately following the initial vortex-mixing. Cells were pelleted by centrifugation at room temperature for 1 min in a Beckman Microfuge B at 9600 *g*. The supernatant fractions which contain extracellular [^{32}P]Pi were removed by aspiration. These fractions were typically clear and colourless, indicating the lack of cell lysis during the incubation. The cell pellet fractions were lysed by vortex-mixing in 0.50 ml of 5 mM-sodium phosphate, pH 8. Proteins were precipitated from the lysate with 0.50 ml of 20% (w/v) trichloroacetic acid, removed by centrifugation, and the supernatant fractions, containing the small molecules retained by cells, were counted for radioactivity in 10 vol. of liquid-scintillation fluor (ACS II, Amersham). Long time points were obtained to determine the equilibrium levels of phosphate transport in non-inhibited samples. Typically, one-third of the added radioactivity was retained by the cells at equilibrium.

The rate of phosphate transport was expressed in terms of the half-time ($t_{1/2}$) to reach equilibrium [9]. In all the experiments reported here, the equilibrium level of phosphate transport in demethylated cells was equal to that in control methylated cells. The $t_{1/2}$ is determined from a semilogarithmic plot of the fraction of transported phosphate versus time. The fraction of transported phosphate is calculated by using the following formula:

$$\text{fraction of transported phosphate outside cells at time } x = 1 - \frac{c.p.m._{t(x)} - c.p.m._{t(0)}}{c.p.m._{t(e)} - c.p.m._{t(0)}}$$

where $c.p.m._{t(0)}$ indicates the cell-retained radioactivity at zero time which represents the background, $c.p.m._{t(e)}$ indicates the value at equilibrium, and $c.p.m._{t(x)}$ indicates the value at time x . Control experiments showed that the phosphate uptake activity of band 3 measured here is inhibited 97% by treatment with 10 μM -DIDS for 15 min at 4 °C, and 97% and 93% by incubation in the presence of 60 mM-sodium sulphanilate or 60 mM-NaCl, respectively [9, 18]. Similar experiments showed that phosphate transport in adenosine- and L-homocysteine thiolactone-treated cells was also inhibited by DIDS (results not shown).

[^3H]Methylation of intact erythrocytes and determination of protein [^3H]methyl ester formation

Packed erythrocytes were incubated with L-[methyl- ^3H]methionine (Amersham; 85 Ci/mmol, final concentration 11.8 μM) in iso-osmotic phosphate, containing 16.7–22.2 mM-glucose, at a 28.6% haematocrit as described elsewhere for 'Condition B' [19]. After shaking for 3 h at 37 °C, cells were pelleted and washed in 200 vol. of iso-osmotic phosphate at 4 °C prior to hypo-osmotic lysis with 5 mM-sodium phosphate, pH 8.0. Membranes were washed at 4 °C with the same buffer. Protein methyl esters were quantified by using an acid precipitation, base hydrolysis, ethanol/toluene extraction, volatilization assay as described [14]. Methylation of individual protein

Table 1. Effect of methylation inhibitors on the half-time of phosphate transport in erythrocytes

Cells were preincubated with glucose for 2 h at 37 °C as described in 'Experimental procedures' and were then incubated for 24 h with or without methylation inhibitors as specified. At the end of the incubation period, cells were washed and resuspended in iso-osmotic phosphate. A portion of the cells was assayed for their ability to incorporate radioactivity from L-[methyl- ^3H]methionine into membrane protein methyl esters. Another portion was assayed for anion transport.

Variation to standard incubation protocol	Half-time of phosphate transport (min)*		Relative number of radioactive methyl esters in membrane proteins†
	Assayed at 37 °C	Assayed at 24 °C	
None (control)	3.03 ± 0.68 (3)	31.3 ± 6.2 (7)	100
Cells not incubated	3.00 ± 0.58 (6)	30.8 ± 7.8 (6)	—
+ 5 mM-Adenosine	3.5 ± 0.7 (2)	30.0	74
+ 5 mM-Homocysteine thiolactone	3.0 ± 0.3 (2)	26.0	66
+ 5 mM-Adenosine and 5 mM-homocysteine thiolactone	5.2 ± 1.6 (2)‡	36.2 ± 4.0 (4)‡	12
+ 50 mM-Cyclolucine	4.1		17
No methionine	2.8		
+ 5 mM-Adenosine and 5 mM-homocysteine thiolactone, no methionine	6.8		
+ 50 mM-Cyclolucine, no methionine	4.0		

* Expressed as the mean ± S.D. The number in parentheses represents the number of experiments. Each experiment represents data from duplicate assays.

† Methyl ester formation was determined by the amount of acid-precipitable, base-labile, ethanol/toluene-extractable, volatile radioactivity in the membrane. The control value of 100 represents 20 pmol of methyl esters/mg of membrane protein.

‡ Significantly different from the control at 80% confidence limits, but not at 90% confidence limits by Student's *t*-test.

species including band 3 was confirmed by SDS/polyacrylamide-gel electrophoresis of the membranes at pH 2.4 and fluorography of the dried gel (cf. [12, 19]; results not shown).

RESULTS

The function of a protein can be altered by covalent modification reactions such as methylation. Reversible modification can regulate protein activity by altering the distribution of modified and unmodified populations of the protein. We were interested in determining if carboxyl methylation can modulate the anion transport activity of band 3 in intact cells. We thus measured the anion transport activity of erythrocytes, using phosphate as a substrate, as a function of the level of band 3 methylation. This level was manipulated in these experiments by incubation of cells with methylation inhibitors [16].

Two separate procedures were used to reduce the level of band 3 methylation in intact cells. The first method involves incubating cells in the presence of adenosine and L-homocysteine thiolactone. The enzymic formation of AdoHcy, a potent methyltransferase inhibitor [20], from 5 mM-adenosine and 5 mM-L-homocysteine thiolactone by intact erythrocytes is very rapid, and the incorporation of methyl esters into all membrane proteins was < 10% that of control cells incubated without the AdoHcy precursors [16]. Since band 3 methyl esters turn over under these conditions with half times ranging from 1 to 15 h [16], it can be calculated that incubation with the methylation inhibitors for 24 h would result in a decrease in the level of methylation in these demethylated cells to less than 20% of that of control cells [16].

An alternative procedure for inhibiting erythrocyte methylation involves inhibiting the synthesis of intracellular AdoMet by incubating intact cells in the presence of cycloleucine, an inhibitor of AdoMet synthetase [16, 21], and/or in the absence of L-methionine, one of the metabolic precursors of AdoMet. Since the endogenous AdoMet pool in erythrocytes turns over rapidly (half-time < 10 min at 37 °C [16]), the inhibition of methylation would be expected to be rapid. Cells that were incubated in either one or both of the above conditions for 24 h should have an increase in the demethylated protein populations, as compared with control cells.

The observed rates of phosphate transport in control and demethylated cells, expressed as the half-time to equilibration, are shown in Table 1. The rate of phosphate transport for cells demethylated by cycloleucine and/or methionine starvation was not significantly different from that of the control cells. On the other hand, there was a marginally significant decrease in phosphate transport in adenosine- and homocysteine thiolactone-treated cells. This decrease was dependent upon the extended incubation of cells under these conditions, because the accumulation of intracellular AdoHcy for a short period (1 h) was not found to affect the anion transport rate (Fig. 1). This result suggests the possibility that the degree of carboxyl methylation may affect the anion transport activity.

If the anion transport activity of band 3 is directly modulated by carboxyl methylation, the degree of anion transport inhibition should be reversed when the methylation inhibitors are removed and the methylation level of band 3 is raised. The reversibility of the inhibition of anion transport was tested when the demethylated

Fig. 1. Rate of phosphate transport as a function of intracellular AdoHcy concentration

Erythrocytes were incubated in standard incubation buffer (see 'Experimental procedures') containing the indicated concentrations of adenosine and L-homocysteine thiolactone for 1 h at 37 °C and were then washed. Phosphate transport was assayed at 24 °C and intracellular AdoHcy concentration was determined by amino acid analysis as described [21].

population of band 3 in adenosine and homocysteine thiolactone-treated cells was allowed to be remethylated. Table 2 shows that when the inhibition of methylation by AdoHcy was reversed by using the adenosine deaminase treatment described previously [16] and cells were allowed to remethylate for 24 h, the rate of phosphate transport was not only not reversed back to the level of control cells, but it continued to decrease. This result shows that the inhibition of anion transport is not a direct consequence of the redistribution of band 3 towards the unmethylated form. Control experiments in which cells were incubated in the presence of adenosine and L-homocysteine thiolactone for a total of 48 h indicate that the rate of anion transport was further reduced, compared with cells incubated with adenosine and L-homocysteine thiolactone for 24 h, although the rate of anion transport was not decreased when control cells were incubated for 48 h.

DISCUSSION

Effect of methylation inhibitors on erythrocyte anion transport

The evidence presented here suggests that inhibition of methylation reactions causes at most small changes in the rate of band 3 catalysed anion transport (Table 1). However, these reactions do not appear to be involved in the immediate regulation of the activity of this transport protein, because reversal of inhibition of methylation does not reverse the change in the rate of anion transport (Table 2). The cause of the small inhibitory effect of adenosine and homocysteine thiolactone incubations on anion transport is not clear. One of the limitations of studies using inhibitors is that these compounds may cause many metabolic and potentially

Table 2. Effect of reversal of methylation inhibition on anion transport by erythrocyte band 3

Cells were incubated with or without adenosine and L-homocysteine thiolactone for 24 h at 37 °C as described in 'Experimental procedures'. At the end of this period, a portion of each sample was incubated with L-[methyl-³H]methionine; a second portion of each was assayed for anion transport at 24 °C (lines 1 and 2). The rest of the sample of adenosine- and L-homocysteine thiolactone-treated cells was incubated for 2 h with a solution of adenosine deaminase as described [16], followed by washing and incubation in fresh buffer with or without adenosine and L-homocysteine thiolactone for an additional 24 h. Methylation and anion transport assays were performed in duplicate at the end of this period (lines 4 and 5). The rest of the samples of control cells were washed and incubated in fresh medium for 24 h and were then used for methylation and anion transport assay (line 3).

Conditions	Relative rate of anion transport (%) [*]	[³ H]Methyl ester formation in membrane proteins (%) [†]
1. Control cells (incubated 24 h)	100	100
2. AdoHcy cells (incubated 24 h)	74	14
3. Control cells (incubated 48 h)	114	76
4. Remethylated AdoHcy cells (incubated 24 h in AdoHcy medium initially, then incubated 24 h in control medium)	64	63
5. AdoHcy cells (incubated 48 h)	50	10

^{*} ($t_{1/2}$ control/ $t_{1/2}$ inhibited) × 100; 100% represents a $t_{1/2}$ control value of 32 min.

[†] Determined by the assay procedures described [14]; 100% represents 20 pmol of methyl ester/mg of membrane protein.

non-physiological changes in the cells. For example, cyclic AMP metabolism has been shown to be altered as a result of incubation of cells under conditions where methyl transfer reactions are inhibited [22]. In addition, the methylation of molecules other than band 3 and other membrane proteins may be involved, since effects of AdoHcy and cycloleucine are not directed exclusively towards the inhibition of protein carboxyl methylation but towards all other AdoMet-dependent methylation reactions as well.

Protein carboxyl methylation and anion transport

The steady state level of carboxyl methylation of band 3 has been estimated to be very low in intact erythrocytes; only a small fraction of molecules appear to be methylated [12, 19]. One might expect that the modification of only a few sites might be of little consequence to the anion transport activity, but it should be pointed out that a significant fraction of band 3 molecules are linked to one another through the membrane cytoskeletal network, and that the methylation of only a few sites in band 3 molecules (or in the connecting cytoskeletal proteins) could affect the activity of many band 3 anion transporters [12].

An alternative role for mammalian protein carboxyl methylation has been proposed recently. The specificity of the erythrocyte protein carboxyl methyltransferase for L-isoadipyl and perhaps D-aspartyl residues in a variety of proteins and peptides suggests that the methylation reaction may be a part of a repair or degradation mechanism for age-damaged aspartyl residues in proteins [1, 23–25]. According to this hypothesis, the function of a protein would not necessarily be altered by the degree of covalent modification *per se*, but the lack of such modification would result in the absence of

protein repair or degradation and may cause the eventual deterioration of protein activity due to an accumulation of the 'damaged' protein population.

The lack of correlation between the anion transport activity and band 3 methylation on a time scale of up to 48 h, as well as the substoichiometry of the modification reaction, point to the conclusion that the primary physiological role of erythrocyte protein carboxyl methylation may not be the regulation of band 3 anion transport activity. The results presented here are not inconsistent, however, with the hypothesis that this modification takes part in a repair or degradation mechanism for damaged proteins.

These studies were supported by a Grant-in-Aid from the American Heart Association (with funds contributed in part by the Greater Los Angeles Affiliate) and by a grant from the National Institutes of Health (GM-26020). L. L. Lou was partially supported by a USPHS Training Grant HL 07386 and S. C. is a Research Fellow of the Alfred P. Sloan Foundation.

REFERENCES

- Clarke, S. (1985) *Annu. Rev. Biochem.* **54**, 479–506
- Kehry, M. R., Bond, M. W., Hunkapiller, M. W. & Dahlquist, F. W. (1983) *Proc. Natl. Acad. Sci. U.S.A.* **80**, 3599–3603
- Terwilliger, T. C. & Koshland, D. E., Jr (1984) *J. Biol. Chem.* **259**, 7719–7725
- Boyd, A., Kendall, K. & Simon, M. I. (1983) *Nature (London)* **301**, 623–626
- Springer, M. S., Goy, M. F. & Adler, J. (1979) *Nature (London)* **280**, 279–284
- Gagnon, C. & Heisler, S. (1979) *Life. Sci.* **25**, 993–1000

7. O'Dea, R. F., Viveros, O. H. & Diliberto, E. J., Jr. (1981) *Biochem. Pharmacol.* **30**, 1163–1168
8. Cabantchik, Z. I. & Rothstein, A. (1974) *J. Membr. Biol.* **15**, 207–226
9. Ho, M. K. & Guidotti, G. (1975) *J. Biol. Chem.* **250**, 675–683
10. Cabantchik, Z. I., Knauf, P. A. & Rothstein, A. (1978) *Biochim. Biophys. Acta* **515**, 239–302
11. Macara, I. G. & Cantley, L. C. (1983) *Cell Membr. Methods Rev.* **1**, 41–87
12. Freitag, C. & Clarke, S. (1981) *J. Biol. Chem.* **256**, 6102–6108
13. Terwilliger, T. C. & Clarke, S. (1981) *J. Biol. Chem.* **256**, 3067–3076
14. O'Connor, C. M. & Clarke, S. (1983) *J. Biol. Chem.* **258**, 8485–8492
15. Galletti, P., Paik, W. K. & Kim, S. (1978) *Biochemistry* **17**, 4272–4276
16. Barber, J. R. & Clarke, S. (1984) *J. Biol. Chem.* **259**, 7115–7122
17. Ramjeesingh, M., Grinstein, S. & Rothstein, A. (1980) *J. Membr. Biol.* **57**, 95–102
18. Passow, H. (1964) in *The Red Blood Cell* (Bishop, C. & Surgenon, D., eds.), pp. 71–145, Academic Press, New York
19. Barber, J. R. & Clarke, S. (1983) *J. Biol. Chem.* **258**, 1189–1196
20. Borchardt, R. T. (1977) in *The Biochemistry of Adenosyl-methionine* (Salvatore, F., Borek, E., Zappia, V., Williams-Ashman, H. G. & Schlenk, F., eds.), pp. 151–171, Columbia University Press, New York
21. Oden, K. L. & Clarke, S. (1983) *Biochemistry* **22**, 2978–2986
22. Zimmerman, T. P., Schmitges, C. J., Wolfberg, G., Deeprose, R. P., Duncan, G. S., Cuatrecasas, P. & Elion, G. B. (1980) *Proc. Natl. Acad. Sci. U.S.A.* **77**, 5638–5643
23. McFadden, P. N. & Clarke, S. (1982) *Proc. Natl. Acad. Sci. U.S.A.* **79**, 2460–2464
24. Murray, E. D., Jr. & Clarke, S. (1984) *J. Biol. Chem.* **259**, 10722–10732
25. Aswad, D. W. (1984) *J. Biol. Chem.* **259**, 10714–10721

Received 5 August 1985/28 October 1985; accepted 27 November 1985