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DIFFERENCES IN COENZYME SPECIFICITY OF THE N^5-METHYLTETRAHYDROFOLATE-HOMOCYSTEINE METHYLTRANSFERASES OF VARIOUS SPECIES: IMPLICATIONS FOR CORRIN BINDING LOCI

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Investigations of the coenzyme specificity of N^5-methyltetrahydrofolate-homocysteine methyltransferases of diverse biological origin revealed previously unrecognized differences between Escherichia coli methyltransferase and the corresponding enzymes of other species. Cyanocobalamin (CNCbl) actively supports methyltransferase in extracts of animal tissues and E. coli. Cobinamide is more active than CNCbl with rat liver methyltransferase; however, it is non-competitively inhibitory with E. coli enzyme. E. coli methyltransferase, but not rat liver enzyme, is competitively inhibited by α-ribazole 3'-phosphate and 5,6-dimethylbenzimidazole, two moieties of the nucleotide loop. This suggests that animal enzyme binds its corrinoid coenzyme at a site on the corrin macro-ring, while E. coli enzyme binds to the nucleotide loop as well as the macro-ring.

In studies of the coenzymatic competence of cobalamin analogues with N^5-methyltetrahydrofolate-homocysteine methyltransferase (EC 2.1.1.13) (hereafter termed methyltransferase), we found that with enzymes from E. coli and rat liver isolated analogues were more active than the four common cobalamins--cyanocobalamin (CNCbl), adenosylcobalamin, (AdoCbl), methylcobalamin and hydroxocobalamin (1). Further studies of the coenzyme specificity of the methyltransferases of various species revealed surprising differences that suggest differing mechanisms of corrin binding.

METHODS

Livers of 250 g adult male rats (Kx strain, New England Deaconess Hospital) were homogenized in 9 vol water and centrifuged at 100,000 × g for 30 min. Escherichia coli B were grown in one-liter cultures of minimal salts and glucose medium for 4 h at 37°C (2). Propionibacterium shermanii (ATCC 9614) were grown without aeration for 18 h at 37°C in a medium containing yeast extract and tryptone (Difco), sodium lactate and minimal salts (as in E. coli cultures) (3). Harvested bacteria were washed and resuspended in 0.05 M potassium phosphate buffer, pH 7.2, disrupted in a cooled MSE ultrasonic disintegrator, and centrifuged at 4,300 × g for 10 min. Aliquots of extract containing 0.1-0.4 mg protein were assayed for N^5-methyltetrahydrofolate-homocysteine methyltransferase according to Peytremann, Thorndike and Beck (4) except that dithiothreitol (Calbiochem), 20 μmol ml^{-1}, replaced β-mercaptoethanol, incubations were conducted under N_2, and corrins

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were added in varying concentrations as indicated. This procedure assays holoenzyme activity in the absence of added corrin and total activity (holo- plus apoenzyme) in the presence of added corrin. Because our interest is in apoenzyme and its possible activity with added analogues, enzyme activity is defined as the difference between activities in incubations with and without added corrin. Holoenzyme activities are noted for reference purposes. One unit of enzyme forms one pmole methionine per min. Boiled enzyme controls were uniformly inert.

RESULTS

When extracts of rat liver and E. coli methyltransferases were compared (Figs. 1A,B), both enzymes were stimulated by added CNCbl; the specific activity of E. coli enzyme was higher than that of rat liver enzyme. However striking differences between the two enzymes were observed when the added corrin was $(CN)_2$Cbi (dicyanocobinamide). Rat liver methyltransferase activity was increased sharply by low concentrations of $(CN)_2$Cbi, reaching higher levels than were achieved with added CNCbl, whereas $(CN)_2$Cbi was inert as a coenzyme for E. coli methyltransferase.

The effects of these corrins on methyltransferase activity in several other tissues are summarized in Table 1. When CNCbl and $(CN)_2$Cbi were added in concentrations of 50 μM--a saturating concentration for CNCbl (8)--similar patterns were observed in all tested animal tissues. As expected (4,9), activities were higher in the presumably proliferating cells of immature rat liver than in those of mature rat liver, both in corrin-supplemented and unsupplemented systems.

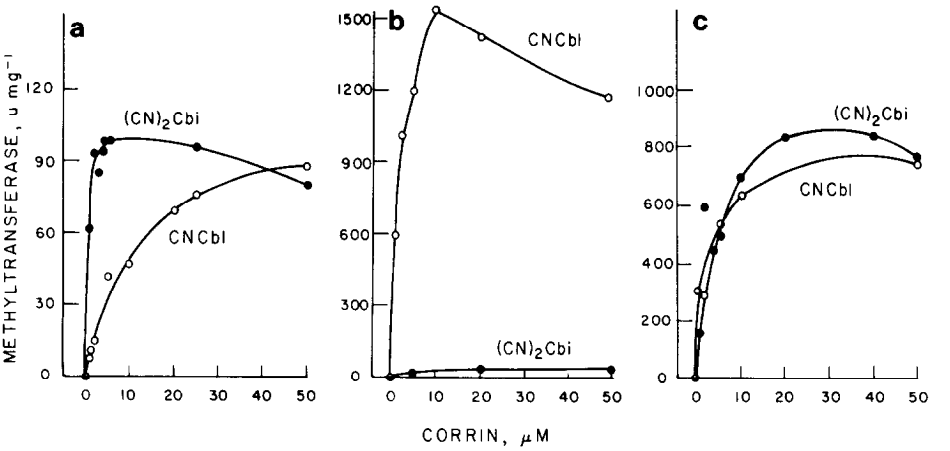

Figure 1. Effect of added CNCbl and $(CN)_2$Cbi on methyltransferase activity in extracts of (a) rat liver, (b) E. coli, and (C) P. shermanii. Activity levels in units mg^{-1} in the absence of added corrin (holoenzyme levels) were 15.6 (rat liver), 19 (E. coli), and 71 (P. shermannii).

Table 1

Coenzymatic activity of CNCbl and $(CN)_2$Cbi with methyltransferases of different species

Source of extract	Methyltransferase activity (u mg^{-1}) in presence of:				
	No addition	CNCbl 5 μM	CNCbl 50 μM	$(CN)_2$Cbi 5 μM	$(CN)_2$Cbi 50 μM
Animal tissues					
Rat liver, adult	18	63	94	132	105
Rat liver, immature	62		244		185
Rat kidney	94	227	309	322	308
Rat pancreas	60		285		233
Human lymphocytes	15		49		33
Bacteria					
Escherichia coli B	17	940	945	<10	<10
Propionibacteria shermanii	74	523	744	381	653
Lactobacillus leichmannii					
Cultivated in dAdo	<10	<10	34	<10	29
Cultivated in CNCbl	<10	<10	50	<10	<10

Liver, kidney and pancreas were obtained from the adult rats described in Fig. 1. Livers were also obtained from 3 day old immature rats. Human blood lymphocytes from a patient with Sézary syndrome, in which methyltransferase is higher than in normal lymphocytes (4), were isolated as previously described (5,6). Lactobacillus leichmannii (ATCC 7830) were grown for 18 h at 37°C in one-liter cultures of Bacto B_{12} Assay Medium (Difco) containing 1 ng ml^{-1} CNCbl or 15 μg ml^{-1} deoxyadenosine (dAdo) (7). Preparation of extracts was as described in Fig. 1 for other bacteria. Data shown are means of 2-10 determinations.

Activity in rat kidney was even higher. In all tested animal tissues, 50 μM $(CM)_2$Cbi was nearly as effective as 50 μM CNCbl in supporting methyltransferase activity.

Patterns differed in extracts of three bacterial species. As noted above, and as earlier observed by Weissbach et al. (10), $(CN)_2$Cbi did not stimulate methyltransferase from E. coli B. However, like animal enzymes, methyltransferase from P. shermannii was actively supported by both CNCbl and $(CN)_2$Cbi. L. leichmannii, a cobalamin- or deoxyribonucleoside-auxotroph (11), displayed little methyltransferase activity that in extracts of deoxyadenosine (dAdo)-grown organisms was weakly stimulated by CNCbl and $(CN)_2$Cbi. An effect by $(CN)_2$Cbi could not be detected in CNCbl-grown organisms, which may be presumed to contain substantial levels of bound cobalamin (12).

The shapes of the curves in Fig. 1A imply notable differences in the affinities of rat liver enzyme for CNCbl and $(CN)_2$Cbi. The curve for $(CN)_2$Cbi is anoma-

lous displaying maximal enzyme stimulation at low coenzyme concentrations (1-5 μM). Double reciprocal plots indicate K_m values of 1.3 μM for CNCbl and 16 nM for $(CN)_2$Cbi--a nearly 100-fold difference. A similar pattern is observed with rat kidney extracts, in which K_m values were 1.1 μM of CNCbl and 83 nM for $(CN)_2$Cbi. Evidently, methyltransferase activity in both rat tissues is actively stimulated by $(CN)_2$Cbi at low concentrations at which CNCbl is only weakly active. Cobalamins assays, performed by the radioisotope dilution method using intrinsic factor as binder (13) or by microbiological assay using Lactobacillus leichmannii (ATCC 7830) (14) (neither assay detects $(CN)_2$Cbi (15)), showed that 50 μM $(CN)_2$Cbi is not converted to cobalamin during rat tissue methyltransferase incubations.

The evidence in Table 1 that $(CN)_2$Cbi inhibits E. coli methyltransferase activity in CNCbl-supplemented incubations, a finding earlier reported by Guest (16), is confirmed by data (Fig. 2A) showing that increasing concentrations of $(CN)_2$Cbi inhibit E. coli methyltransferase activity in the presence of 1 μM CNCbl.

Since cobinamide is an intermediate of cobalamin synthesis in Propionibacteria and other microorganisms (18,19), it is of interest that $(CN)_2$Cbi actively

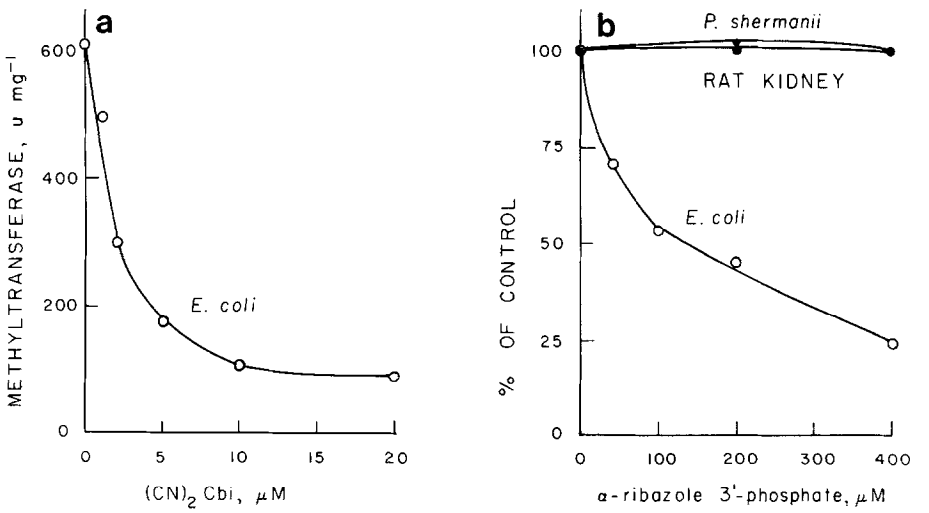

Figure 2. (a) Inhibition of E. coli methyltransferase by $(CN)_2$Cbi. As described in the text, activities in the absence of added corrinoid were subtracted from observed activities. (b) Inhibition of E. coli methyltransferase, but not of rat liver or P. shermannii methyltransferase, by α-ribazole 3'-phosphate (3,17). Activities in the presence of 1 μM CNCbl (without added α-ribazole 3'-phosphate) are set equal to 100%. Absolute values in units mg^{-1} were 640 (E. coli), 155 (rat kidney), and 248 (P. shermannii).

supports methyltransferase activity in P. shermanii (Table 1). However, as shown in Fig. 1C, $(CN)_2$Cbi is not more potent at low concentrations than CNCbl, as it is with rat liver methyltransferase (Fig. 1A).

The striking difference in the capacities of rat liver and E. coli methyltransferases to function with $(CN)_2$Cbi suggests that the two enzymes bind to different sites on the corrin molecule. Since cobinamide lacks the lower structure that has been termed the "nucleotide loop," rat liver enzyme would appear to bind its coenzyme at a site on the corrin macro-ring, whereas E. coli methyltransferase presumably binds to the nucleotide loop as well as the macro-ring. Thus it would be anticipated that compounds representing moieties (or their analogues) of the nucleotide loop, such as α-ribazole 3'-phosphate or 5,6-dimethylbenzimidazole, might occupy binding sites on the E. coli enzyme, but not on the rat liver enzyme, and competitively inhibit the CNCbl-dependent methyltransferase activity of E. coli, but not of rat tissue. When the hypothesis was tested, an inhibitory effect of α-ribazole 3'-phosphate on CNCbl-dependent E. coli methyltransferase was evident (Fig. 2B). Other experiments show comparable inhibition by 5,6-dimethylbenzimidazole (200 μM). Ribose 5-phosphate was inert when added in similar concentrations to E. coli methyltransferase. This may explain the old observation that 5,6-dimethylbenzimidazole (and other benzimidazoles) are growth inhibitors of various cobalamin-dependent bacterial mutants (20). The double

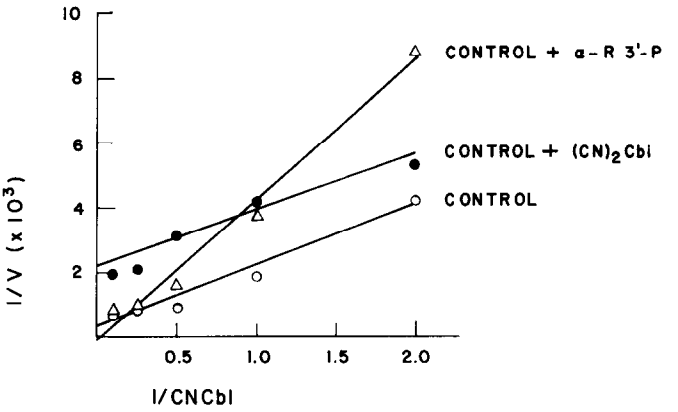

Figure 3. Double reciprocal least squares plots showing effects of CNCbl concentration on activity of E. coli methyltransferase in presence of 5 μM $(CN)_2$Cbi or 200 μM α-ribazole 3'-phosphate.

reciprocal plots in Fig. 3 suggest that in inhibiting E. coli methyltransferase α-ribazole 3'-phosphate competes with CNCbl (approximate K_i, 200 μM) and $(CN)_2$Cbi acts non-competitively. There was no inhibition of the methyltransferases of rat kidney or P. shermanii.

DISCUSSION

The results imply that the binding of its coenzyme by E. coli methyltransferase involves sites on the nucleotide loop and the corrin macro-ring, whereas the methyltransferases of animal tissues (and perhaps Propionibacteria) bind only the corrin macro-ring. Since $(CN)_2$Cbi inhibition of E. coli enzyme is non-competitive with CNCbl, the two corrinoids may occupy different sites on the enzyme. Probably the methyltransferase of animal cells has only a single binding site for corrinoid coenzymes, but that point is not established by these results.

These observations echo the familiar fact that R protein (perhaps in analogy with rat liver methyltransferase) binds both corrinoids while intrinsic factor (like E. coli methyltransferase) binds only cobalamin (13). However, we failed in preliminary experiments to demonstrate inhibition by α-ribazole 3'-phosphate of cobalamin binding by intrinsic factor.

Since cobinamide, an intermediate of cobalamin synthesis, is fully competent as a coenzyme of methyltransferase in P. shermanii, a question arises as to why the organism troubles to complete the synthesis of cobalamin. The probable answer is that this species also contains an AdoCbl-dependent methylmalonyl CoA mutase (21) and perhaps other cobalamin-dependent enzymes that cannot function with cobinamide.

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