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THE DEFECT IN THE cbl B CLASS OF HUMAN METHYLMALONIC ACIDEMIA: DEFICIENCY OF COB(I)ALAMIN ADENOSYLTRANSFERASE ACTIVITY IN EXTRACTS OF CULTURED FIBROBLASTS

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SUMMARY: We show that the reductants present in the in vitro assay used to measure the formation of adenosylcobalamin from cob(III)alamin by cell-free extracts of human fibroblasts result in the non-enzymatic reduction of cob(III)alamin to cob(I)alamin. Hence, the in vitro assay uniquely estimates the activity of ATP:cob(I)alamin adenosyltransferase (EC 2.5.1.17). Based on additional studies with extracts of fibroblasts from patients in the cbl B class of human methylmalonic acidemia and from their parents, we conclude that this mutant class results from a specific deficiency of adenosyltransferase activity which is inherited as an autosomal recessive trait.

Inherited methylmalonic acidemia in man may be due either to defects in the methylmalonyl CoA mutase apoenzyme or to deficiencies in the intracellular synthesis of adenosylcobalamin (AdoCbl)*, the coenzyme form of cobalamin (Cbl; vitamin B_{12}) required for methylmalonyl CoA mutase activity (1). Fibroblasts from patients in the latter category comprise four biochemically and genetically distinct classes or complementation groups (cbl A, cbl B, cbl C, cbl D) (2). The class designated cbl B is characterized by deficient conversion of cob(III)alamin (Cbl^{III}), usually supplied as hydroxocobalamin (OH-Cbl), to AdoCbl not only by intact fibroblasts in culture, but also by cell-free extracts prepared therefrom (3).

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*Abbreviations: Cbl, cobalamin; Cbl^{III}, cob(III)alamin; Cbl^{II}, cob(II)alamin; Cbl^{I}, cob(I)alamin; OH-Cbl, hydroxocobalamin; AdoCbl, adenosylcobalamin; CMCbl, carboxymethylcobalamin.

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By analogy with bacterial systems, AdoCbl synthesis in man probably involves three enzymatic steps: two one-electron reductions catalyzed by cob(III)alamin reductase and cob(II)alamin reductase (Eq. 1), followed by an ATP-requiring adenosylation by ATP:cob(I)alamin adenosyltransferase (Eq. 2) (4-6).

$$Cbl^{III} + e^- \rightarrow Cbl^{II} + e^- \rightarrow Cbl^{I} \qquad 1.$$

$$Cbl^{I} + ATP \rightarrow AdoCbl + PPP_i \qquad 2.$$

When Mahoney and his colleagues reported the in vitro biochemical characterization of the cbl B class, they were unable to assign the defect to a specific enzyme because they could not ascertain which of the reductive steps was bypassed non-enzymatically by their assay system (3). They suggested, based on previous chemical studies of the reduction of OH-Cbl by thiols (7), that at least the cob(III)alamin reductase had been circumvented by the inclusion of dithiothreitol in the assay; they were unable to determine the role of the cob(II)alamin reductase, however.

We report here our investigation of the possibility that both reductive steps (Eq. 1 above) are accomplished non-enzymatically in the assay. To do this, we have taken advantage of the fact that the most reduced species, cob(I)alamin (Cbl^I), is an extremely powerful nucleophile, reacting rapidly, even at low concentrations, with alkyl halides to form stable alkylcobalamins (Eq. 3) (8).

$$Cbl^{I} + R\text{-}X \rightarrow R\text{-}Cbl + X^- \qquad 3.$$

This reaction permits the trapping of the highly unstable Cbl^I in a form which can be isolated by thin-layer chromatography and, when ^{57}Co-labeled Cbl is the starting material, quantitated by scintillation spectrometry (9). In addition, because cob(II)alamin (Cbl^{II}) does not react with alkyl halides (8), the roles of the two reduced forms of Cbl can be distinguished. Iodoacetate has been used as the alkyl halide in this study because it is soluble in aqueous solutions and because the alkylcobalamin derived from it, carboxymethylcobalamin (CMCbl), is

easily separated from other cobalamins by thin-layer chromatography.

In addition to separating the enzymatic and non-enzymatic components of the assay, we have estimated adenosyltransferase activity in fibroblast extracts from several cbl B mutants not previously reported and from both parents of a cbl B mutant.

METHODS

Materials. CN-[^{57}Co]Cbl (200-300 Ci/mmol) was obtained from Amersham and converted to OH-[^{57}Co]Cbl as previously described (9). Other reagents were obtained from the usual commercial sources. Human fibroblast cell lines (identified by our laboratory's cell accession numbers) were maintained in Eagle's minimal essential medium with 10% fetal bovine serum and were harvested with trypsin-EDTA as described (3). Cell-free extracts were prepared by brief sonication, followed by centrifugation for 30 min at 40,000 x g.

Trapping and quantitation of cob(I)alamin as CMCbl. The reduction and trapping were carried out in a Thunberg tube with a single side arm by a modification of the published AdoCbl synthesis assay (3). The main body of the tube contained: 1.1 pmol OH-[^{57}Co]Cbl (85,000 cpm/pmol), 0.10 μmol FAD, 50 μmol potassium iodoacetate, and 100 μmol potassium phosphate, pH 7.4, in a total volume of 0.85 ml; the side arm contained 50 μmol dithiothreitol or 0.5 μmol NADH in 0.25 ml. Cell extract (up to 1 mg protein), ATP (1.0 μmol), and $MgCl_2$ (0.8 μmol) were added to the main body of the tube when appropriate. The tube was closed and its contents deoxygenated by alternate evacuation and flushing with hydrogen gas (about ten cycles). The reaction was started by tipping the reductant into the main body of the tube and placing the tube at 37°C. After 30 min, the reaction was stopped by opening the tube to the atmosphere and applying its contents to a silanized silica gel column (RP-2; E. Merck); the column was washed with 1% acetic acid, and then the cobalamins were eluted with 50% methanol/1% acetic acid as described (10). Authentic unlabeled OH-Cbl and CMCbl (10 nmol each) were added to the eluate; and, after concentration by evaporation, the eluate was applied to a Baker-Flex IB2 thin-layer plate (J.T. Baker). The plate was developed with 2-butanol:2-propanol:water:NH_4OH (50:50:50:1) as described (9); CMCbl migrates with an R_f of 0.62 under these conditions, as compared to R_f's of 0.45 for AdoCbl and 0.05 for OH-Cbl. The colored regions corresponding to OH-Cbl and CMCbl were cut from the plates and their content of [^{57}Co]Cbl determined by a Packard Auto-Gamma spectrometer.

Cob(I)alamin adenosyltransferase assay. AdoCbl synthesis catalyzed by cob(I)alamin adenosyltransferase was estimated under conditions similar to those above, except that iodoacetate was omitted from the reaction and ATP, $MgCl_2$, and cell extract were present. Column and thin-layer chromatography were performed identically, with 10 nmol unlabeled AdoCbl added instead of CMCbl.

All operations involving CMCbl and AdoCbl were carried out under severely reduced illumination. Protein was determined by the method of Lowry et al. (11).

RESULTS AND DISCUSSION

Tables 1 and 2 summarize the results obtained from a number of experiments defining the components of the AdoCbl synthesis assay necessary for trapping Cbl^{I} as CMCbl. The complete system, with either dithiothreitol or NADH as reductant (Table 1, expts. 1 and 2a), produces a significant amount of Cbl^{I} from Cbl^{III}, approximately 20% of the starting material being converted in 30 min. When either the reductant (Table 1, expt. 2b) or the flavin nucleotide (Table 1, expt. 2c) are omitted, no detectable Cbl^{I} is formed. These requirements are identical to those reported for *in vitro* AdoCbl synthesis (3). The presence of oxygen (Table 1, expt. 3) and the substitution of Cbl^{II} for Cbl^{III}, FAD and NADH (Table 1, expt. 4) both result in undetectable Cbl^{I} formation.

Importantly, the inclusion of normal fibroblast extract (Table 2, expt. 2a) produces no increase in the amount of Cbl^{I} formed as measured by trapping with iodoacetate. On the other hand, when ATP is substituted for iodoacetate (Table 2, expt. 3), the presence of cell extract supports AdoCbl synthesis; about the same amount of AdoCbl is formed as there is Cbl^{I} generated (in Table 2, compare expt. 3 with expt. 1a). Finally, when ATP, iodoacetate and cell extract are present (Table 2, expt. 2b), the non-enzymatic trapping reaction competes effectively with the enzymatic adenosylation reaction, resulting in reduced AdoCbl synthesis and substantial CMCbl formation.

Taken together, these data clearly show that OH-Cbl can be reduced non-enzymatically to Cbl^{I} by NADH (or dithiothreitol) in the presence of FAD to an extent sufficient to account for the enzymatic synthesis of AdoCbl when a source of adenosyltransferase and ATP are present. Thus, it is clear that the assay used by Mahoney et al. (3) and others (12) to estimate AdoCbl synthesis *in vitro* is specific for ATP:cob(I)alamin adenosyltransferase. This in turn implies that the cell lines of the *cbl B* class, which Mahoney et al. described as deficient in AdoCbl

Table 1. Conditions for the non-enzymatic reduction of Cbl^{III} to Cbl^{I}

Experiment	Reaction conditions	Cbl^{I} formed (pmol CMCbl/hr)
1	Complete* (dithiothreitol)	0.42
2a	Complete (NADH)	0.43
2b	Complete - NADH	<0.02
2c	Complete - FAD	<0.02
3	Complete (aerobic)+	<0.02
4	Complete - Cbl^{III}, -NADH, -FAD, +Cbl^{II}¶	<0.02

*The "complete" reaction contained buffer, Cbl^{III}, reductant (dithiothreitol or NADH), FAD, and iodoacetate, incubated anaerobically, as detailed in the text. Cbl^{I} was trapped, and the CMCbl formed was quantitated as described; dithiothreitol was used as the reductant only in Expt. 1.

+The reaction tube was not evacuated and flushed with hydrogen.

¶[^{57}Co]Cbl^{II} was generated *in situ* by the anerobic photolysis of Ado[^{57}Co]Cbl.

Table 2. Role of cell extract in formation of Cbl^{I} and AdoCbl

Experiment	Reaction conditions	Product formed (pmol/hr)	
		CMCbl	AdoCbl
1a	Complete*	0.48	-¶
1b	Complete + ATP	0.36	<0.02
2a	Complete + cell extract+	0.42	-
2b	Complete + cell extract + ATP	0.47	0.12
3	Complete - iodoacetate + cell extract + ATP	-	0.40

*The "complete" reaction contained buffer, Cbl^{III}, NADH, FAD, and iodoacetate, incubated anaerobically. Cbl^{I} was trapped as CMCbl as before.

+An aliquot of a control fibroblast extract, containing 0.5 mg protein, was added.

¶"-" means "none expected".

Table 3. Cob(I)alamin adenosyltransferase activity in fibroblast extracts

Cell line	Adenosyltransferase activity (pmol AdoCbl formed/hr per mg protein)
87 (control)*	1.26
105 (control)*	1.41
237 (control)*	0.99
1f (cbl A)†	1.25
245 (cbl A)†	1.28
224 (cbl B)†	<0.02
500 (cbl B)	<0.02
563 (cbl B)	<0.02
487 (cbl B)	<0.02
488 (mother of 487)	0.36
489 (father of 487)	0.38

Adenosyltransferase activity was determined as in text. Means of duplicate determinations are reported. Aliquots of extracts containing 0.2-0.4 mg protein were assayed for the control and cbl A lines; up to 2 mg of extract protein was added for the assay of cbl B lines and those of the parents.

*Three different control lines were used, each of them multiple times; representative values are reported. The mean for all determinations using control lines was 1.26 pmol/hr per mg protein.

†These lines have been reported previously (3).

synthesis (3), are, in fact, specifically deficient in adenosyltransferase activity.

Having established that the assay is specific for adenosyltransferase, we have proceeded to confirm the previously reported biochemical characterization of several cell lines of the cbl B class and to examine several recently obtained lines assigned to the cbl B class solely on the basis of genetic complementation analysis. Table 3 contains these results. In each case, the extract from the cbl B cell line has undetectable adenosyltransferase activity when compared to either a

control cell extract or an extract from a cell line from the cbl A complementation group. None of the extracts examined (from control, cbl A, or cbl B lines) has any effect on the non-enzymatic reduction of Cbl^{III} to Cbl^{I} (data not shown).

Table 3 also shows data obtained from adenosyltransferase assays on extracts of fibroblasts derived from the mother (line 488) and father (line 489) of a child expressing the cbl B defect (line 487). The fact that both parents have reduced (~30% of control mean) adenosyltransferase activity implies that they are heterozygous for the mutant allele and is consistent with the limited data from pedigree analysis that the cbl B defect is inherited as an autosomal recessive trait.

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