First publ. in: Journal of Biological Chemistry 256 (1981), 19, pp. 9809-9812

Communication

Inactivation of General Acyl-CoA Dehydrogenase from Pig Kidney by a Metabolite of Hypoglycin A*

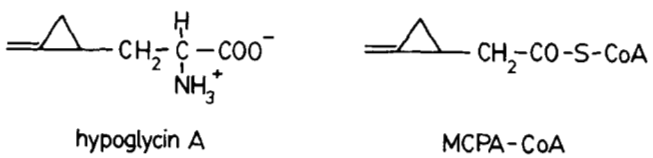
Alexandra Wenz‡, Colin Thorpe§¶, and Sandro Ghisla‡‖

From the ‡Fakultät fur Biologie der Universität Konstanz, Postfach 5560, D-7750 Konstanz, West Germany and the §Department of Chemistry, University of Delaware, Newark, Delaware 19711

Pig kidney general acyl-CoA dehydrogenase is irreversibly inactivated by methylenecyclopropylacetyl-CoA, a metabolite of the hypoglycemic amino acid hypoglycin from *Blighia sapida*, to less that 2% of native activity. Octanoyl-CoA affords strong protection against this inhibition. During inactivation, about 80% of the enzyme FAD is covalently and irreversibly modified with the residual inhibition possibly resulting from modification of the protein. Denaturation of the inactivated enzyme yields several modified flavin derivatives in addition to about 20% unmodified FAD. From spectral comparison, the structure of one of these species is tentatively assigned to a derivative of 4α,5-dihydroflavin, while two further products resemble 6-, and 8-substituted flavins. These results suggest that methylenecyclopropylacetyl-CoA (and consequently the methylenecyclopropylmethano moiety of hypoglycin) be considered "suicide" substrates.

Hypoglycin, an unusual amino acid, is the active and toxic component of the unripe ackee fruit (from *Blighia sapida*). While the ripe fruit serves as a dietary staple in Jamaica, ingestion of its unripe arillus causes severe hypoglycemia and often death in man (1, 2). Hypoglycin was first isolated by Hassall and Reyle (3) and was later found to be metabolized to methylenecyclopropylacetate in mammals (4). The latter has been implicated in at least some of the toxic effects (1), and its CoA-ester is believed to inhibit oxidation of fatty acids both *in vivo* (5) and *in vitro* (6, 7). While the physiological and pharmacological effects of hypoglycin have received appropriate attention (8), the molecular mechanism of action is not so well understood.

The salient chemical feature of hypoglycin is the methylenecyclopropyl moiety linked to the β position of an alanine residue (Scheme 1). The chemical properties of such a group led us to suspect that it might constitute the dormant function of a "suicide inhibitor." In fact, α,β oxidation of fatty acids is probably initiated by proton abstraction at the α position of a CoA-ester (9, 10), generating a transient carbanion, which then serves to transform the methylenecyclopropyl function into a highly reactive intermediate. The same consideration would also apply to the methylenecyclopropylglycin, the lower homolog of hypoglycin, which is found in the seeds of litchi fruit and which also exhibits hypoglycemic activity in mammals (11).



hypoglycin A MCPA-CoA

SCHEME 1. **Structures of hypoglycin A and methylenecyclopropylacetyl-CoA.**

In addition to the pharmacological and physiological relevance of hypoglycin and of its metabolites, the study of the interaction of MCPA[1]-CoA with the acyl-CoA dehydrogenases would be expected to complement investigation of the mode of dehydrogenation of normal substrates. Analogous studies with flavin-dependent α-hydroxy acid dehydrogenases/oxidases (12–15) have been particularly fruitful.

MATERIALS AND METHODS

Pig kidney general acyl-CoA dehydrogenase was purified and assayed as described previously (16). Enzyme concentrations refer to bound FAD (ϵ_{446} = 15.4 mM^{-1} cm^{-1}). Octanoyl-CoA and CoA (Li^+) were from PL Biochemicals. Phosphodiesterase (*Crotalus adamanteus*) was from Sigma.

Hypoglycin from ackee fruit was a generous gift from Dr. C. H. Hassall and was converted to MCPA by enzymatic degradation with *L*-amino acid oxidase (*Crotalus atrox*; Serva, Heidelberg) (17) and H_2O_2. The product was characterized by NMR and mass spectroscopy and was pure by thin layer chromatography (Merck silica gel plates; cyclohexane/2-propanol, 4:1). Thioesterification of MCPA with CoA was by a modification of the procedure of Goldman and Vagelos (18). The thioester compound was characterized by ^{1}H-NMR (purity according to CoA- *versus* acyl-residue proton integration $\geq$ 80%), and thin layer chromatography (silica gel; butanol/acetic acid/water, 5:2:3; one spot observable by nitroprusside reaction after alkaline hydrolysis). The concentrations of the thioesters were determined using 2,2′-dithiopyridine after hydrolysis with 0.1 M NaOH for 30 min at 37 °C (19) and from ultraviolet spectra at pH 7 before and after hydrolysis under the same conditions (ϵ_{260} (adenine), ~15,400 M^{-1} cm^{-1} and ϵ_{232} (thioester), ~9,400 M^{-1} cm^{-1}).

Three methods were used to release flavin from the modified enzyme. 1) The enzyme was treated with 5% trichloroacetic acid at 4 °C. After centrifugation, the supernatant was extracted with ether and neutralized with solid sodium bicarbonate to pH 7. 2) The enzyme solution was brought to 80% methanol, allowed to stand for 12 h at 4 °C and centrifuged; or 3) the enzyme solution was heated to 100 °C for 5 min and centrifuged. The solutions obtained in these methods were then concentrated by flash evaporation or lyophilization.

High pressure liquid chromatography was performed using a μBondapak C_{18} column, a programmable Kontron gradient generator, and Altex pumps. One hundred-μl aliquots were generally applied and eluted with a linear 5 to 40% gradient of methanol and 20 mM phosphate buffer, pH 6.0. The eluates were monitored at 260 nm using a Kontron UVIKON LC 720. Peak areas were calculated using a Hewlett Packard 3390A Integrator.

RESULTS

Fig. 1 shows the inactivation of general acyl-CoA dehydrogenase by a 4-fold molar excess of MCPA-CoA at pH 7.6. About 90% of the enzyme activity is lost rapidly, followed by

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

¶ Recipient of National Institutes of Health Grant GM 26643.

‖ Recipient of a Deutsche Forschungsgemeinshaft grant.

[1] The abbreviations used are: MCPA, methylenecyclopropylacetate; HPLC, high pressure liquid chromatography.

Konstanzer Online-Publikations-System (KOPS)
URL: http://www.ub.uni-konstanz.de/kops/volltexte/2008/5730/
URN: http://nbn-resolving.de/urn:nbn:de:bsz:352-opus-57301

a slower decline to a residual activity of less than 2% at 2 h. Octanoyl-CoA affords marked protection against this inactivation (*e.g.* at 0, 1.0, and 1.5 equivalents of substrate, inactivation is half-complete in about 1, 10, and 40 min, respectively; Fig. 1). Enzyme maintained anaerobically in the reduced state in the presence of 300 μM dithionite is not significantly inactivated by MCPA-CoA.

The spectral changes which accompany inactivation are shown in Fig. 2. The final spectrum (*curve d*) shows extensive bleaching of the oxidized flavin chromophore with bands at 320, 360, 380, and 450 nm and a very weak absorbance extending to 700 nm. Prolonged storage of the modified enzyme under aerobic or anaerobic conditions or dialysis does not produce further spectral changes and catalytic activity is not restored. Although the spectral changes depicted in Fig. 2 were obtained under both anaerobic and aerobic conditions, the stoichiometry of inactivation differs by a factor of about 2 (*inset*), suggesting that the inhibitor partitions approximately equally between turnover and inactivation. It should be emphasized that MCPA-CoA has been reported to be very unstable and was previously generated *in situ* (20). The material isolated in this work proved similarly difficult to handle, and this lability could account for the stoichiometry observed here being greater than 1. The *inset* to Fig. 2 (*solid symbols*) also shows that the extent of flavin modification, assessed by the irreversible decrease in 446 nm absorbance under aerobic conditions, parallels the loss of enzyme activity.

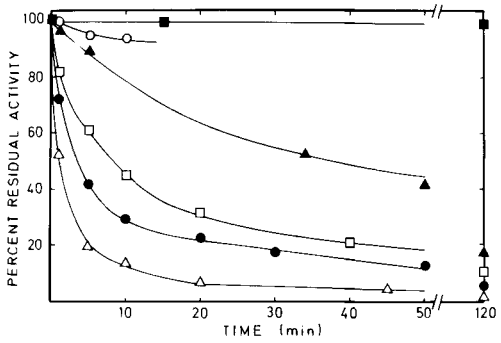


FIG. 1. **Inactivation of general acyl-CoA dehydrogenase by MCPA-CoA.** General acyl-CoA dehydrogenase (6.8 μM) in 50 mM phosphate buffer, pH 7.6, was incubated at 25 °C with 27 μM MCPA-CoA and 0.0 (△), 3.4 (●), 6.8 (□), 10.2 (▲), and 20 μM octanoyl-CoA (○). A control incubation (■) showed no significant lost in activity.

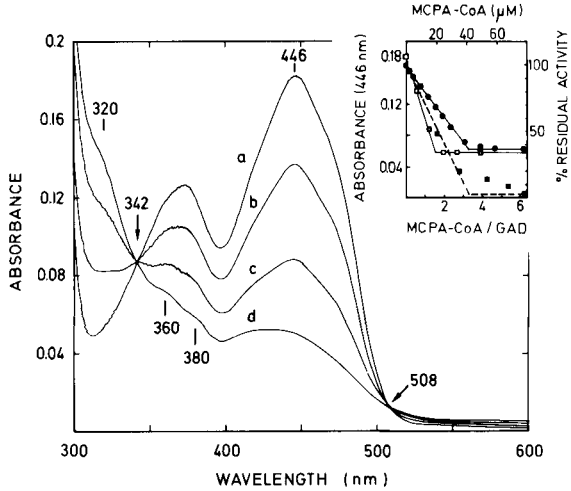


FIG. 2. **Effect of MCPA-CoA on the spectrum of general acyl-CoA dehydrogenase.** The enzyme, 12 μM in 0.1 M phosphate buffer, pH 7.6 (*curve a*), was titrated anaerobically with 8, 16, and 48 μM MCPA-CoA (*curves b–d,* respectively). Intermediate spectra were recorded after absorbance changes were completed (about 10 min), and *curve d* represents the spectrum obtained on completion of the titration. Isosbestic points are indicated by *arrows.* The *inset* shows the 446 nm absorbance values plotted *versus* the amount of MCPA-CoA added under anaerobic conditions (□) and anaerobic conditions (●). The latter *points* should be compared with aerobic titration monitored by enzyme activity (■) assayed 10 min after each successive addition of MCPA-CoA. GAD, general acyl-CoA dehydrogenase.

A careful comparison of the absorption spectra of native general acyl-CoA dehydrogenase and inactivated enzyme after extensive dialysis or gel filtration reveals an increased absorption of the latter in the 260 nm region, corresponding to approximately one equivalent of CoA-adenine being covalently incorporated into the enzyme (0.9 ± 0.15 equivalents/FAD in three separate experiments).

As shown above, MCPA-CoA-treated general acyl-CoA dehydrogenase exhibits a spectrum with three absorption shoulders in the near UV and a maximum at ~450 nm (Fig. 2). Upon denaturation of the protein by trichloroacetic acid ("Materials and Methods"), the absorption shoulders disappear, and a spectrum with a maximum at 390 nm and a pronounced shoulder at ~450 nm is formed. In contrast, denaturation with 2% sodium dodecyl sulfate, with methanol, or by heat treatment does not cause major modifications of the inactivated general acyl-CoA dehydrogenase spectrum. An HPLC analysis of the products released on denaturation is shown in Table I. Peak A has a retention time very similar to normal FAD and is not detected in the trichloroacetic acid extract. The spectrum of this nonfluorescent chromophore at

TABLE I

HPLC analysis of compounds released on denaturation of MCPA-CoA inactivated general acyl-CoA dehydrogenase

Method of denaturation	Relative peak area of eluted products[a]							
	CoASH	$(CoAS)_2$	FAD	A	B	C	D	Area[b]
				%				
Methanol	3	5	15	15	15	20	23	4 (5)
Heat	25	11	22	16	9	6	1	10 (4)
Trichloroacetic acid		14	14		10	36	3	23 (7)
Retention time (in min ± 0.5 min)	11.5	17.5	24	25	29	31	33	

[a] Compounds eluting with retention times <10 min, which were in part identified, *e.g.* trichloroacetic acid or AMP, and compounds eluting after 36 min were not considered in the calculation of the relative peak area. Their area was <13% of the total area; see "Materials and Methods" for further details.

[b] Total area of the several minor peaks observed with their numbers in parentheses.

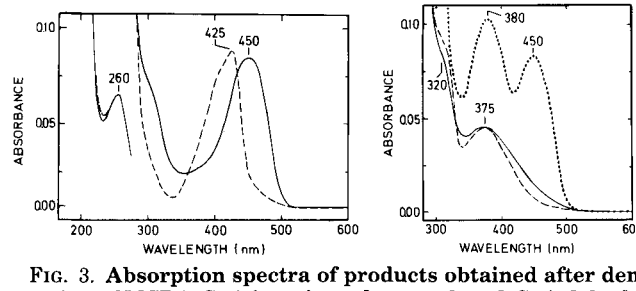


FIG. 3. **Absorption spectra of products obtained after denaturation of MCPA-CoA inactivated general acyl-CoA dehydrogenase.** The compounds were separated as described under "Materials and Methods." *Left,* absorption spectra of compound A in 20 mM phosphate buffer, pH 6.0 (——); and pH 0 (- - -). *Right,* absorption spectra at pH 6.0 of compounds B (——) and C (· · · ·); (- - -) shows the spectra of the model coenzyme 4*a*,5-propano-4*a*,5-dihydro-FMN taken from Ref. 13).

neutral pH and upon protonation (Fig. 3) are strikingly similar to the corresponding spectra of 8-0-R substituted flavins (21). Peak B, a nonfluorescent product obtained by all methods of denaturation, has a spectrum closely similar to that of 4*a*,5-disubstituted flavins such as 4*a*,5-propano-4*a*,5-dihydro FMN (Fig. 3, *right*) and to that of the primary product of inactivation of lactate oxidase from *Mycobacterium smegmatis* by α-hydroxybutynoic acid (12, 13). Its structure is thus tentatively proposed to be derived from a 4*a*,5-dihydroflavin chromophore. The spectrum of peak C, the predominant product of trichloroacetic acid denaturation, but also present in the other extracts, has a fluorescence emission maximum at 525 nm, which is increased 3-fold on treatment with phosphodiesterase (in the case of normal FAD, an 8-fold increase is observed (22)). Its spectrum (Fig. 3, *right*) is reminiscent of 6-substituted flavins (23) and is partially bleached by dithionite. The constituent of peak D is present mainly in the methanol extract and has a maximum absorbance at 260 nm, with a pronounced shoulder at 300 nm, an absorption spectrum often encountered with degradation products of 4*a*,5-disubstituted flavins (12). The several other minor peaks, obtained by all denaturation methods, were found to have no characteristic absorbance in the visible or near UV region and probably constitute secondary decay products.

DISCUSSION

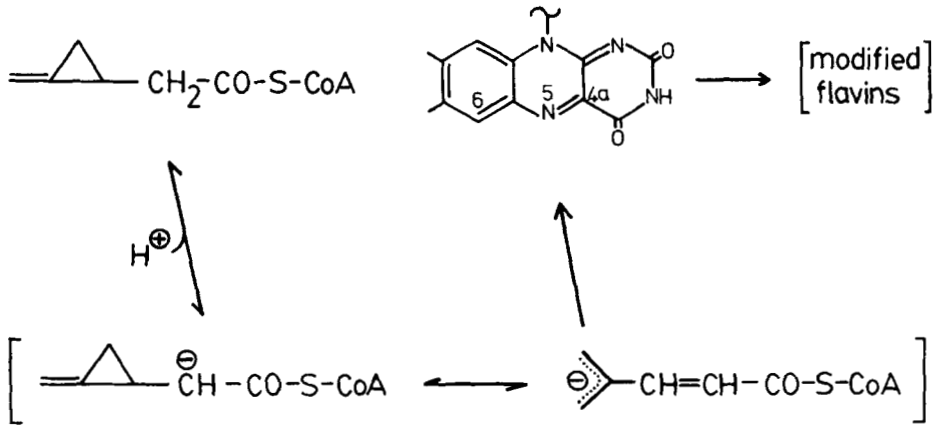
The spectral modification obtained upon incubation of general acyl-CoA with MCPA-CoA (Fig. 2) and the analysis of the modified chromophores obtained upon protein denaturation (Table I) clearly establish that the flavin coenzyme has been covalently modified concomitant with inactivation. The spectral changes at 260 nm obtained upon inhibition are consistent with the incorporation of ~1 mol of adenine/enzyme flavin. CoA degradation products, which must derive from the original MCPA-CoA inhibitor, are also found in varying amounts in the supernatant of denatured inactivated general acyl-CoA dehydrogenase (Table I). Although modification of the flavin coenzyme of general acyl-CoA dehydrogenase is obviously the major inactivation pathway, the existence of a minor pathway involving alkylation of the protein cannot yet be ruled out. Indeed, addition of dithionite to the modified enzyme, exhibiting less than 2% residual activity, leads to a further 15–20% decline in 450 nm absorbance from the final spectrum shown in Fig. 2. A corresponding amount of unmodified FAD is also detected after denaturation (Table I). Clarification of the possible importance of protein modification must await the use of radiolabeled MCPA-CoA.

Elucidation of the structures of the modified flavin derivatives is underway and is considerably more complicated than that encountered with lactate oxidase inactivated with α-hydroxybutynoic acid (12 ,13). As adducts of basically different structural types are found in the HPLC analyses, it is unlikely that they arise from a single common precursor. In agreement with this, mild methanol denaturation does not cause major changes in the spectrum of inactivated general acyl-CoA dehydrogenase; however, HPLC analysis reveals several distinct chromophores. While component B can reasonably be assigned to a 4*a*,5-dihydroflavin derivative, the structures of A and C cannot be confidently predicted at present. Nevertheless, it is hoped that eventual elucidation of the structures of compounds A–C will provide insights into the mechanism of action of the acyl-CoA dehydrogenases.

A number of investigators have suggested that abstraction of an α-proton is an early step in the dehydrogenation of acyl-CoA thioesters (9, 24–26). In the present case, due to the peculiarities of the methylenecyclopropane moiety, generation of a transient α-carbanion would be expected to lead to ring opening and covalent addition to the flavin electrophile (Scheme 2). Such a mechanism has been proposed for the isomerization of MCPA-esters by alkali to yield 4-methyl-2,4-pentadienoyl esters (27). Thus, we suggest that MCPA-CoA should be considered as a suicide, "k_{cat}," "mechanism-based," or "Trojan horse" inhibitor according to the criteria discussed elsewhere (28–32). Indeed, the course of inactivation and, in particular, the formation of covalent flavin adducts, parallels that encountered with other suicide inhibitors of flavin enzymes. The toxic effects of hypoglycin may thus be ascribed, at least in part, to such a type of inhibition. It is noteworthy that the related saturated compound methylcyclopropylalanine, which would be less efficient in the stabilization of an incipient α-carbanion, does not show hypoglycemic activity (11).

It should be noted that the effects of MCPA-CoA are not restricted to acyl-CoA dehydrogenases of general specificity, since similar spectral changes to those reported here are observed on inactivation of butyryl-CoA dehydrogenase from *Megasphera elsdenii* (32). Recently, 3-butynoyl-, 3-octynoyl-, and 2,3-octadienoyl-CoA have been shown to inhibit liver general acyl-CoA dehydrogenase by irreversible modification of the protein (26), in clear distinction to the flavin modification reactions described in this work. In addition, Gomes *et al.* have recently reported the inhibition of the bacterial butyryl-CoA dehydrogenase by 3-butynoylpantetheine and conclude that an α-carbanion mechanism is the most probable pathway for inhibition and catalysis (25).

Acknowledgments—We wish to thank Dr. C. H. Hassal for a generous gift of hypoglycin A and Drs. P. Engel and P. Hemmerich for fruitful discussions.



SCHEME 2. **Possible mechanism of inactivation of acyl-CoA dehydrogenase by MCPA-CoA.**

REFERENCES

1. Tanaka, K. (1979) in *Handbook of Clinical Neurology* (Vinken, P. J., and Bruyn, G. W., eds) Vol. 37, pp. 511–539, Elsevier/North Holland, Amsterdam
2. Sherratt, H. S. A. (1969) *Br. Med. Bull.* **25,** 250–255
3. Hassall, C. H., and Reyle, K. (1955) *Biochem. J.* **60,** 334–339
4. von Holt, C. (1966) *Biochim. Biophys. Acta* **125,** 1–10
5. von Holt, C., von Holt, M., and Bohm, H. (1966) *Biochim. Biophys. Acta* **125,** 11–21
6. Manchester, K. L. (1974) *FEBS Lett.* **40,** 133–139
7. Kean, E. (1976) *Biochim. Biophys. Acta* **422,** 8–14
8. Tanaka, K. (1972) *J. Biol. Chem.* **247,** 7465–7478
9. Cornforth, J. W. (1959) *J. Lipid Res.* **1,** 1–28
10. Walsh, C. T. (1980) *Acc. Chem. Res.* **13,** 148–155
11. Gray, D. O., and Fowden, L. (1962) *Biochem. J.* **82,** 385–389
12. Schonbrunn, A., Abeles, R. H., Walsh, C. T., Ghisla, S., Ogata, H., and Massey, V. (1976) *Biochemistry* **15,** 1798–1807
13. Ghisla, S., Ogata, H., Massey, V., Schonbrunn, A., Abeles, R. H., and Walsh, C. T. (1976) *Biochemistry* **15,** 1791–1797
14. Olson, S. T., Massey, V., Ghisla, S., and Whitfield, C. D. (1979) *Biochemistry* **18,** 4724–4732
15. Abeles, R. H., and Maycock, A. L. (1976) *Acc. Chem. Res.* **9,** 313–319
16. Thorpe, C., Matthews, R. G., and Williams, C. H. (1979) *Biochemistry* **18,** 331–337
17. Tanaka, K. (1972) *J. Biol. Chem.* **247,** 7465–7478
18. Goldman, P., and Vagelos, R. P. (1961) *J. Biol. Chem.* **236,** 2620–2629
19. Grassetti, D. R., and Murray, J. F. (1967) *Arch. Biochem. Biophys.* **119,** 41–49
20. Osmundsen, H., and Sherratt, H. S. A. (1975) *FEBS Lett.* **55,** 38–41
21. Ghisla, S., and Mayhew, S. G. (1976) *Eur. J. Biochem.* **63,** 373–390
22. Bessey, O. A., Lowry, O. H., and Love, R. H. (1949) *J. Biol. Chem.* **180,** 755–769
23. Mayhew, S. G. (1971) *Biochim. Biophys. Acta* **235,** 289–302
24. Hemmerich, P. (1964) in *Mechanismen Enzymatisches Reaktionen (Mosbach Symposium)* pp. 183–209, Springer Verlag, Berlin
25. Gomes, B., Fendrich, G., and Abeles, R. H. (1981) *Biochemistry* **20,** 1481–1490
26. Frerman, F. E., Miziorko, H. M., and Beckman, J. (1980) *J. Biol. Chem.* **255,** 11192–11198
27. Ullman, E., and Fanshawe, W. J. (1961) *J. Am. Chem. Soc.* **83,** 2379–2383
28. Walsh, C. T. (1978) *Annu. Rev. Biochem.* **47,** 881–893
29. Abeles, R. H. (1978) in *Enzyme-activated Irreversible Inhibitors* (Seiler, N., Jung, M. J., and Koch-Weser, J., eds) pp. 1–12, Elsevier/North-Holland, Amsterdam
30. Rando, R. R. (1978) in *Enzyme-activated Irreversible Inhibitors* (Seiler, N., Jung, M. J., and Koch-Weser, J., eds) pp. 13–26, Elsevier/North-Holland, Amsterdam
31. Miesowicz, F. M., and Bloch, K. (1979) *J. Biol. Chem.* **254,** 5868–5877
32. Ghisla, S., Wenz, A., and Thorpe, C. (1980) in *Enzyme Inhibitors* (Brodbeck, U., ed) pp. 43–60, Verlag Chemie, Weinheim West Germany