A Reappraisal of the Reaction of Butyryl-Coenzyme A Dehydrogenase with Phenylmercuric Acetate

EVIDENCE THAT DE-GREENING INVOLVES A REACTION OF THE TIGHTLY BOUND THIOESTER

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Phenylmercuric acetate reversibly de-greens butyryl-CoA dehydrogenase from *Megasphaera elsdenii*, abolishing the absorption band at 710 nm. The view that this is a result of modification of a protein thiol group is re-examined in the light of the following new observations. (i) After treatment with phenylmercuric acetate, the enzyme's ability to be re-greened by addition of thiols was not decreased by gel filtration or precipitation with $(\text{NH}_4)_2\text{SO}_4$. (ii) Phenylmercuric acetate caused the same extent of de-greening whether added in a few large amounts or many small ones. The overall time taken for de-greening was, however, greatly extended when many small additions were made. (iii) In Tris/acetate buffer, pH 7.5, 3.5 mol of phenylmercuric acetate/mol of enzyme subunit was required for complete de-greening, compared with only 2.5 mol/mol in phosphate buffer, pH 7. (iv) None of the groups that react with phenylmercuric acetate is accessible to iodoacetate or iodoacetamide. (v) On a molar basis dithiothreitol, mercaptoethanol and CoA are equally effective in re-greening the enzyme. (vi) Provided that phenylmercuric acetate is not present in excess, the de-greened enzyme forms normal and stable complexes with crotonyl-CoA and acetoacetyl-CoA. (vii) When a small excess of phenylmercuric acetate is present, full stable development of the enzyme-acetoacetyl-CoA complex requires addition of several mol of acetoacetyl-CoA/mol of enzyme subunit. (viii) The ability of de-greened enzyme to be immediately re-greened by an excess of thiol declines with time, more rapidly at pH 6 than at pH 7 or 8, but at all three pH values the instantaneous re-greening was followed by a slow phase of further increase in A_{710} . This further recovery was most extensive and most rapid at pH 8. These findings are reminiscent of the previously described reversible decline in the re-greening capacity of a protein-free acid extract of green butyryl-CoA dehydrogenase. It is concluded that the likely cause of de-greening is chemical modification of the tightly bound thioester rather than a protein thiol group. The reversibility would be explained if the thioester exists on the surface of the enzyme in equilibrium with free CoA and a lactone, or if the acyl group is readily and reversibly transferred from the thiol of CoA to a protein side chain.

The short-chain fatty-acyl CoA dehydrogenase (EC 1.3.99.2), as normally isolated from mammalian or bacterial sources, exhibits a broad absorption band centred at 710 nm which is responsible for the striking green colour of this flavoprotein (Green *et al.*, 1954; Mahler, 1954; Steyn-Parvé & Beinert, 1958*b*; Hoskins, 1966; Engel & Massey, 1971*a*). The attribution of this colour to a copper prosthetic group (Mahler, 1954) was shown to be incorrect by Steyn-Parvé & Beinert (1958*b*) who suggested instead that the absorption band was due to an interaction between the protein and the bound FAD. Palmer & Massey (1968) proposed that an anionic cysteine

residue might serve as the donor in a charge-transfer complex with FAD, and suggested that the effect of mercurials on the enzyme should be investigated. Subsequently, Engel & Massey (1971*b*) obtained evidence suggesting that the missing partner in the postulated charge-transfer complex was a tightly bound acyl-CoA molecule, and drew attention to the analogy with the complexes formed by succinate dehydrogenase (Veeger *et al.*, 1966) and D-amino acid oxidase (Massey & Ganther, 1965) with certain competitive inhibitors. They also, however, investigated the effects of various compounds that react with thiol groups. Among these,

phenylmercuricacetate, 5,5'-dithiobis-(2-nitrobenzoic acid) and CuSO_4 caused 'de-greening', converting the absorption spectrum into that of a normal flavoprotein (Engel & Massey, 1971a). *N*-Ethylmaleimide has now also been added to this list (P. C. Engel, unpublished work).

The effect of phenylmercuric acetate could have been explained in terms of reaction either with a protein thiol group, as originally suggested by Palmer & Massey (1968), or with the bound CoA thioester (Cecil & McPhee, 1959). The latter possibility was not entertained, however, mainly because the effect of phenylmercuric acetate was reversed by adding dithiothreitol. If the thioester had been split by the mercurial, such reversal would imply spontaneous re-formation of the thioester bond. This is not normally feasible because of the high free energy of hydrolysis of thioesters. It was therefore concluded that blockage of a protein thiol group somehow prevented interaction between the moieties responsible for the long-wavelength absorption.

This conclusion, apparently inescapable at the time, nevertheless left certain puzzling aspects of the effect of phenylmercuric acetate unexplained. Surprisingly, for instance, the treatment with mercurial did not affect catalytic activity (Engel & Massey, 1971a). Moreover, the enzyme's capacity to be re-greened by dithiothreitol declined over a period of hours, but again this decline was not accompanied by any change in catalytic activity.

More recent findings (Engel, 1972) have suggested that the tightly bound acyl-CoA may be somewhat atypical in its chemical properties, and have prompted the present reinvestigation of the effects of thiol reagents.

Experimental

The growth of anaerobic cultures of *Peptostreptococcus elsdenii* (renamed *Megasphaera elsdenii*; Rogosa, 1971) and the purification of butyryl-CoA dehydrogenase from the cell paste were carried out essentially as described by Engel & Massey (1971a). Much of the enzyme used in this study was obtained from large-scale cultures (500 litres) grown at the Microbiological Research Establishment, Porton Down, Wiltshire, U.K. The early purification steps were carried out on a large scale at Porton. Enzyme in a state of approx. 50% purity, eluted from DEAE-cellulose, was shipped in ice to Sheffield for further purification by $(\text{NH}_4)_2\text{SO}_4$ fractionation and column chromatography on DEAE-cellulose. The final product was essentially homogeneous butyryl-CoA dehydrogenase as judged by disc gel electrophoresis on polyacrylamide gels and by spectral criteria, although it was not all in the green form (cf. Engel & Massey, 1971a). The

percentage of the yellow form present was usually between 25 and 40%.

Measurements of the absorption spectrum of the enzyme were made with a Cary 14 scanning spectrophotometer and matched silica cuvettes of 1 cm light-path and 1 ml nominal volume. The absorption coefficients used in calculating the concentrations of the green and yellow forms of the enzyme and of the enzyme-acetoacetyl-CoA complex are taken from Engel & Massey (1971a,b).

Except where experiments involved the preparation of a single large sample of enzyme to be divided into portions for different treatment, de-greening and re-greening were carried out in the cuvette in order to avoid losses in the transfer of solutions.

Crotonyl-CoA was prepared by the method of Simon & Shemin (1953) and determined by measuring the thiol released on alkaline hydrolysis with 5,5'-dithiobis-(2-nitrobenzoic acid) (Ellman, 1959). Acetoacetyl-CoA was made as described by Michal & Bergmeyer (1963) and assayed by the method of Stern *et al.* (1956).

CoA was obtained from Boehringer Corp. (London), Lewes, East Sussex BN7 1LG, U.K. Diketen, iodoacetic acid and iodoacetamide were from Aldrich Chemical Co., Gillingham, Dorset SP8 4JL, U.K. The diketene was vacuum-distilled before use and kept under N_2 . Crotonic anhydride and mercaptoethanol were purchased from Eastman Organic Chemicals, Kirkby, Liverpool, U.K. Phenylmercuric acetate was supplied by Hopkin and Williams, Chadwell Heath, Essex RM1 1HA, U.K. Sephadex G-15 was from Pharmacia (G.B.) Ltd., London W5 5SS, U.K. Dithiothreitol, enzyme-grade $(\text{NH}_4)_2\text{SO}_4$ and buffer salts were obtained from British Drug Houses, Poole, Dorset BH12 4NN, U.K.

Results

Effect of $(\text{NH}_4)_2\text{SO}_4$ precipitation on re-greenability

Amino acid analysis of native butyryl-CoA dehydrogenase and of acid extracts from it (Engel & Massey, 1971b) revealed the presence of a pantotheine moiety, providing part of the evidence that the green form of the enzyme has a tightly bound acyl-CoA molecule at the active site. Against expectation, similar analysis of enzyme treated with phenylmercuric acetate and dialysed (Engel & Massey, 1971b) showed no significant decline in the CoA content. This suggests that, unlike acid extraction or anaerobic dialysis of the reduced enzyme, de-greening with the mercurial does not completely displace acyl-CoA from the enzyme. The following experiment provides an independent test of this.

Butyryl-CoA dehydrogenase (A_{710}/A_{430} 0.37), containing approx. $0.28 \mu\text{mol}$ of enzyme-bound flavin,

in 3 ml of 0.1M-potassium phosphate, pH7, was treated with 1.3 μ mol of phenylmercuric acetate dissolved in 0.36 ml of water. The A_{710} declined from an initial value of 0.33 before adding the mercurial to 0.05 after 16 min at room temperature (approx. 20°C). At this point, although de-greening was still incomplete, 1 ml was withdrawn and added to 5 ml of saturated $(\text{NH}_4)_2\text{SO}_4$ solution to precipitate the protein. The sample was centrifuged at 30 000g for 10 min, and the resulting clear colourless supernatant was removed. The pellet of precipitated protein was redissolved in 1 ml of 0.2M-potassium phosphate, pH6.8, giving a yellow solution, which showed a slightly higher A_{710} than before precipitation. Then 0.2 ml of 7.2 mM-dithiothreitol was added, producing an immediate rise in A_{710} from 0.1 to 0.19. Allowing for dilution, this represents 75% of the absorbance expected for full return of the long-wavelength absorbance band. The spectral ratios were remeasured after centrifugation at 30 000g for 10 min to remove a small amount of turbidity, and indicated that the fraction of enzyme in the green form was 72% of the value for the original sample, as compared with only 16% before precipitation with $(\text{NH}_4)_2\text{SO}_4$. If the de-greening by phenylmercuric acetate was due to total displacement of a ligand of low molecular weight, one might have expected very little re-greening by dithiothreitol after the $(\text{NH}_4)_2\text{SO}_4$ precipitation.

Gel filtration of butyryl-CoA dehydrogenase after de-greening with phenylmercuric acetate

In several experiments samples of enzyme were passed down columns of Sephadex G-15 after de-greening by phenylmercuric acetate. Initially this was done in the hope of isolating a displaced compound of low molecular weight responsible for the absorbance band of the native enzyme at 710 nm. In a typical experiment with 0.05 M-Tris/acetate, pH 7.5, as the buffer, the control sample, de-greened at the same time and stored while the main portion was applied to the column, showed ratios of A_{710}/A_{430} of 0.011 before re-greening and 0.164 after re-greening. The peak enzyme fraction eluted from the column showed ratios of 0.049 before, and 0.174 after, re-greening. The result shows two things: firstly it confirms the result of the $(\text{NH}_4)_2\text{SO}_4$ experiment suggesting that the mercurial does not displace the re-greening factor from the surface of the enzyme; secondly, the slight but significant re-greening on the column before addition of thiol indicates that gel filtration removes some of the mercurial from the enzyme.

Titration of green butyryl-CoA dehydrogenase with phenylmercuric acetate under various conditions

Butyryl-CoA dehydrogenase has 5 half-cystine residues per subunit (Engel & Massey, 1971a), and

the fact that up to 4 mol of mercurial/mol of enzyme subunit are required to de-green the enzyme completely (Engel & Massey, 1971a) indicates that several thiol groups are accessible to the reagent. The kinetics of de-greening is complex, and addition of 2–3 mol of phenylmercuric acetate/mol of enzyme subunit leads to initial de-greening followed by partial or sometimes total recovery of the green colour (Engel & Massey, 1971a). This suggests that kinetic differences in the accessibility of thiol groups to the mercurial do not entirely parallel the thermodynamic differences that determine the position of equilibrium. To explore this further, the

Fig. 1. Titrations of green butyryl-CoA dehydrogenase with phenylmercuric acetate in different buffers (a) In 0.1 M-potassium phosphate buffer, pH 7. For details see the text. (b) In 0.05 M-Tris/acetate buffer, pH 7.5. For details see the text.

reaction of phenylmercuric acetate with the enzyme was studied under different conditions.

Fig. 1(a) shows the combined results of three separate experiments with identical samples of butyryl-CoA dehydrogenase (A_{710}/A_{430} 0.36), 100 μM with respect to enzyme subunits, in 0.1M-potassium phosphate, pH 7. In two experiments phenylmercuric acetate was added in several small amounts from a 1 mg/ml solution. After each addition the contents of the cuvette were mixed, and A_{710} was monitored until it became constant. All readings are corrected for dilution. In the first experiment (●) A_{710} was recorded after the addition of 0.5 mol (8 min), 1.0 mol (32 min), 1.5 mol (141 min), 2.0 mol (201 min), 2.2 mol (214 min), 2.4 mol (225 min), 2.6 mol (237 min), 2.8 mol (257 min) and 3.0 mol (267 min)/mol of enzyme-bound FAD. The time shown in parentheses is the total time elapsed between the first addition of mercurial and the measurement of A_{710} at equilibrium for a given point on the graph. After 282 min the enzyme was re-greened with an excess of mercaptoethanol (final reading indicated by the arrow). In the second experiment (▲) A_{710} was recorded after addition of 0.5 mol (9 min), 1.0 mol (22 min), 1.2 mol (42 min), 1.4 mol (106 min), 1.6 mol (210 min), 1.8 mol (242 min), 2.0 mol (274 min), 2.5 mol (307 min) and 3.0 mol (336 min). Both of these titrations showed that the first mol of mercurial added per mol of enzyme subunit caused only 5% de-greening. In the region of the titration curve between 1 and 1.6 mol/mol, after each addition of mercurial, A_{710} decreased sharply at first and then recovered very slowly to the final value recorded on the graph. After addition of the second mol per mol, only 23% of the initial A_{710} remained at equilibrium. At its steepest point, the titration curve has a slope corresponding to complete de-greening by 1 mol of mercurial per mol of enzyme subunit. In considering this stoichiometry, however, it must be borne in mind that the initial enzyme samples were only partly in the green form (approx. 58% green and 42% yellow).

In the third experiment in Fig 1(a) (■ and broken line) the enzyme was de-greened with a single addition of 3 mol of mercurial/mol of enzyme subunit to exactly the same extent as that achieved by adding the same amount of mercurial in several small doses over a much longer period of time. The major difference, however, was in the extent to which the samples could be re-greened. The third sample recovered 72% of its initial A_{710} on addition of 15 μl of 10 mM-mercaptoethanol 28 min after the addition of phenylmercuric acetate (see arrow). The first sample, however, could be re-greened only to 58% of the initial absorbance 280 min after the first addition of mercurial. This confirms the earlier finding (Engel & Massey, 1971a) that the ability of sample to be re-greened declines with time.

Fig. 1(b) shows the results of similar experiments in 0.05M-Tris/acetate, pH 7.5. The concentration of enzyme subunits was 104 μM (A_{710}/A_{430} 0.31). In experiment A (▲) A_{710} was recorded after addition of 3 mol (35 min), 3.5 mol (71 min), 4.0 mol (93 min), 4.5 mol (114 min), or 5.0 mol (150 min)/mol of enzyme subunit. An identical sample de-greened with a single addition of 3 mol of mercurial/mol was treated after 25 min with an excess of mercaptoethanol: A_{710} is shown by a triangle and arrow. In experiment B (●) A_{710} was recorded after addition of 0.5 (3 min), 1.0 (8 min), 1.5 (21 min), 2.0 (49 min) and 3.0 (60 min) mol of mercurial/mol. In experiment C (■) A_{710} was recorded after addition of 2.0 (69 min), 2.2 (78 min), 2.4 (87 min), 2.6 (123 min), 2.8 (150 min) and 3.0 (178 min) mol of mercurial/mol, and after 180 min this sample was re-greened with mercaptoethanol (■ and arrow). In experiment D (no separate symbol because points overlap others), A_{710} was measured after addition of 2.0 (35 min) and 3.0 (45 min) mol of mercurial/mol. All readings are corrected for dilution. Broken lines show the change in A_{710} for single additions of 1 mol of mercurial/mol or more. In this buffer higher concentrations of the mercurial were required for complete de-greening, about 3.5 mol/mol as compared with 2.5 mol/mol in phosphate, pH 7. Correspondingly the maximum slope of the titration curve with Tris/acetate buffer indicates complete de-greening by an increment of 1.5 mol of mercurial/mol. It appears that in this buffer there is greater overlap between the affinities of different reactive groups for the mercurial.

Less detailed experiments, with Tris/acetate, pH 7, as the buffer, indicate that the differences between the titration curves in Figs. 1(a) and 1(b) stem mainly from the change in buffer substance rather than the altered pH; the titration curve with Tris/acetate, pH 7, as the buffer is very similar to that with Tris/acetate, pH 7.5.

Titration with phenylmercuric acetate were unaltered by prior incubation of the enzyme in Tris/acetate, pH 7.5, for 30 min with either 2 mM-iodoacetate or 2 mM-iodoacetamide. It has been shown previously that these compounds do not de-green the enzyme (Engel & Massey, 1971a). The present findings indicate that none of the groups modified by phenylmercuric acetate are accessible to iodoacetate or iodoacetamide.

Studies of specificity with respect to the thiol used for re-greening

In view of the ineffectiveness of certain thiol-specific reagents in de-greening the enzyme, it was decided to find out whether there is similar selectivity for re-greening agents. It was of especial interest to discover whether CoA re-greens more effectively than do other thiol compounds. To

this end, in each of three separate experiments 80 nmol of green butyryl-CoA dehydrogenase (A_{710}/A_{430} 0.38) in 1 ml of 0.1 M-potassium phosphate buffer, pH 7, was de-greened by the addition of 300 nmol of phenylmercuric acetate. After 12 min at room temperature the full visible absorption spectrum was recorded. At this point A_{710} had declined from 0.38 to 0.05. Re-greening by the addition of small volumes of concentrated solutions of thiol compounds was started 16 min after the addition of mercurial. Fig. 2 shows the very similar results obtained with CoA, 2-mercaptoethanol and dithiothreitol. In view of subsequent results, it is possible that the shape of the re-greening titration curve might have been altered if A_{710} had been followed for a longer time after each addition of thiol. This does not, however, affect the conclusion that, in bringing about instantaneous re-greening, the three thiol compounds are equally effective on a molar basis. It is noteworthy that dithiothreitol evidently removes only 1 mol of mercurial/mol of dithiol.

Effect of treatment with phenylmercuric acetate on formation of enzyme-acyl-CoA complexes

After treatment with phenylmercuric acetate, butyryl-CoA dehydrogenase retains full activity (Engel & Massey, 1971a). It seemed surprising that a disturbance sufficient to abolish the absorption band at 710 nm should not also affect catalytic activity. The ability of the enzyme to form recognizable complexes with externally added acyl-CoA compounds after treatment with the mercurial was therefore tested.

From the de-greened enzyme sample used for the

Fig. 2. Re-greening of butyryl-CoA dehydrogenase by different thiols after de-greening with phenylmercuric acetate

After de-greening with phenylmercuric acetate three separate but identical enzyme samples were progressively re-greened with additions of mercaptoethanol (●), dithiothreitol (▲) or CoA (■). For details see the text.

experiment with $(\text{NH}_4)_2\text{SO}_4$ (described above) a further 1 ml was withdrawn and mixed with 50 μl of 2.5 mM-crotonyl-CoA. The absorption spectrum immediately showed the characteristic features of the enzyme-crotonyl-CoA complex (Steyn-Parvé & Beinert, 1958b; Engel & Massey, 1971b), namely a shift of the 450 nm flavin absorbance peak to a longer wavelength and the appearance of pronounced shoulders at 430 and 480 nm.

In a separate experiment 74.7 nmol (in terms of subunits) of butyryl-CoA dehydrogenase (A_{710}/A_{430} 0.34) in 1 ml of 0.05 M-Tris/acetate, pH 7.5, was de-greened by the addition of 238 nmol of phenylmercuric acetate (3.2 mol/mol of enzyme subunit) in 0.08 ml of water (Fig. 3). In 20 min the percentage of the enzyme in the green form, as indicated by the A_{710} , decreased from 63% to 10% (Fig. 3). At this stage 88 nmol of acetoacetyl-CoA (10 μl) was added. The grey-green colour of the enzyme-acetoacetyl-CoA complex was immediately seen, and the spectrum showed the strong absorbance band at 580 nm (Fig. 3) which characterizes this complex (Engel & Massey, 1971b). The spectral ratio (A_{580}/A_{430} 0.22) indicated that about two-thirds of the enzyme

Fig. 3 Formation of a complex between acetoacetyl-CoA and butyryl-CoA dehydrogenase after de-greening with phenylmercuric acetate

The absorption spectrum of the green form of the enzyme at the start of the experiment is shown by the solid line. The dotted line shows the spectrum 20 min after addition of 3.2 mol of phenylmercuric acetate/mol of enzyme subunit. Addition of 1.18 mol of acetoacetyl-CoA/mol of enzyme subunit to the de-greened enzyme gave rise immediately to the spectrum shown by the broken line. For further details see the text.

had been converted into the acetoacetyl-CoA complex.

De-greening with phenylmercuric acetate therefore does not alter the enzyme in such a way as to prevent the formation of apparently normal and stable complexes with crotonyl-CoA and acetoacetyl-CoA. This finding is entirely consistent with the full catalytic activity observed after treatment with phenylmercuric acetate (Engel & Massey, 1971a), but is difficult to reconcile with the view (Engel & Massey, 1971a) that the mercurial reacts with a strategically positioned protein thiol group in the vicinity of the active site.

Stability of the enzyme-acetoacetyl-CoA complex as a function of the amount of mercurial added to the enzyme

Under the conditions of de-greening in Fig. 3, the A_{710} was not entirely abolished before addition of acetoacetyl-CoA, and comparison with Fig. 1(b) suggests that under these conditions (3.2 mol of mercurial/mol of enzyme subunit) very little of the mercurial would remain free in solution. In subsequent experiments larger amounts of mercurial were used to achieve fuller removal of the band at 710 nm. Under these conditions a different pattern was observed on addition of acetoacetyl-CoA. For example, in an experiment with 0.1 M-potassium phosphate buffer, pH 7, in which the enzyme had been de-greened by the addition of 4 mol of mercurial/mol of enzyme subunit, addition of 1 mol of acetoacetyl-CoA/mol of enzyme subunit caused a rapid rise in A_{580} of roughly the expected magnitude, but, after reaching a maximum, the absorbance again fell. Surprisingly, the second mol of acetoacetyl-CoA added per mol of enzyme subunit also failed to give full stable development of the absorption band at 580 nm. Only after addition of 4 mol/mol was this achieved. It must be concluded that, of the 4 mol of thioester added/mol, 3 mol had been used to strip mercurial off the enzyme, giving rise, presumably, to acetoacetate and phenylmercury-CoA mercaptide (Cecil & McPhee, 1959), leaving 1 mol of tightly bound mercurial still on the enzyme and the fourth mol of acetoacetyl-CoA at the active site. Similar instability of the acetoacetyl-CoA complex was seen also in Tris/acetate buffer, pH 7, 7.5, 8 and 8.5, when 4–5 mol of mercurial/mol was used to de-green the enzyme. It is not clear why the complex should be so much more stable under the conditions of Fig. 3.

pH studies of re-greenability as a function of time

As discussed above, after treatment with phenylmercuric acetate, butyryl-CoA dehydrogenase can be re-greened by thiols, but the extent to which the initial A_{710} can be regained declines with time.

The tentative explanation of this decline in terms of a slow rearrangement of the protein structure (Engel & Massey, 1971a) seems unconvincing, since, even when the enzyme has lost all ability to re-green at 710 nm, it is still fully active in the catalytic assay (Engel & Massey, 1971a). In a further attempt to explain the decline in re-greenability, the effect of varying the pH was investigated.

For each of the experiments illustrated in Fig. 4, 195 nmol of enzyme (in terms of subunits) in 0.5 ml of 0.05 M-Tris/acetate buffer, pH 7.5, was de-greened at room temperature by the addition of 594 nmol of phenylmercuric acetate in 0.2 ml of water. After about 20 min 0.6 ml of the de-greened enzyme solution was withdrawn and mixed with 4.2 ml of 1 M-potassium phosphate at pH 6 (Fig. 4a), pH 7 (Fig. 4b) or pH 8 (Fig. 4c). Immediately on mixing, and at intervals thereafter, samples (0.8 ml) of the diluted enzyme solution were taken and mixed with 15 μ l of a 10 mg/ml solution of CoA, used in this experiment as the re-greening agent. The visible-absorption spectra of these samples were recorded immediately on adding the CoA and then again periodically over several hours. In Figs. 4(a), 4(b), and 4(c) the broken curves trace the decline in the instantaneous re-greenability of the three aging samples of de-greened enzyme. The continuous curves indicate the subsequent behaviour of each re-greened sample.

All three experiments document the previously observed decline in instantaneous re-greenability. This decline is most marked at pH 6; the sample kept at this pH regained only 34% of the initial A_{710} (allowing for dilution) after 4 h, compared with over 50% for both the other samples. A new and striking observation, however, is the further recovery of A_{710} after the initial instantaneous re-greening. At pH 7 and pH 8 this further recovery entailed the regain of as much as 25% of the initial A_{710} in addition to that recovered immediately on addition of CoA. This gradual recovery has not been seen previously, because at pH 7 it is relatively slow. At pH 6 it appears to be even slower, whereas at pH 8 it is much faster. At pH 8, moreover, the first two samples taken regained, within experimental error, all their original A_{710} . At pH 6, by contrast, none of the samples at any stage reached a higher A_{710} than that seen in the first sample immediately after adding CoA. The first sample taken at pH 6 actually showed a further decline in A_{710} after addition of the CoA, the second remained steady, and only those that had declined to give instantaneous re-greening of less than 70% showed slow recovery to a higher value. Clearly a reversible process is in part responsible for the decline in re-greenability with time in the absence of added thiol. The results for the sample at pH 6 suggest that on addition of the thiol the system may be poised on either side of an equilibrium

depending on the extent of the previous decline. Superimposed on a reversible change, however, there appears to be also an irreversible decline at all three pH values studied. At pH 8, for example, after 4.25 h the extent of instantaneous re-greening is 53%, rising to a final value of only 66%.

Discussion

Various $\alpha\beta$ -unsaturated and 3-oxoacyl-CoA compounds are bound tightly by the oxidized yellow form of butyryl-CoA dehydrogenase from *Megaspheera elsdenii*. Some of the resulting complexes display absorption bands of large amplitude between 500 and 900 nm (Engel & Massey, 1971b), which are almost certainly due to a charge-transfer interaction with the flavin (Abramovitz & Massey, 1976). It seems very likely that a similar acyl-CoA compound is responsible for the green colour of the naturally occurring form of the enzyme as isolated from many different sources, and direct evidence for its presence has been obtained (Engel & Massey, 1971b). To explain the de-greening action of phenylmercuric acetate and certain other thiol reagents, Engel & Massey (1971a) suggested that the blocking of a thiol group at the active site disrupts the intimate association between the acyl-CoA ligand and the FAD prosthetic group. Several lines of evidence now appear to argue against this simple hypothesis.

(i) The ability of the de-greened enzyme to form normal complexes with crotonyl-CoA and acetoacetyl CoA, showing typical long-wavelength absorption in the latter case, seems incompatible with a bulky substitution at the active site;

(ii) The fact that the complex pattern of declining re-greenability documented in Figs. 4(a)–4(c) is not

Fig. 4. Re-greenability of butyryl-CoA dehydrogenase as a function of time at different pH values

For the three experiments shown in (a), (b) and (c) enzyme was de-greened at pH 7.5 by the addition of 3.05 mol of phenylmercuric acetate/mol of enzyme subunit. When de-greening was nearly complete (after 18 min for a, 29 min for b and 20 min for c) the enzyme was diluted in 1 M-potassium phosphate at pH 6 (a), 7 (b) or 8 (c). The experimental point on the ordinate in each graph indicates the amount of re-greening achieved by adding an excess of CoA to a portion of the enzyme immediately after the dilution with 1 M-potassium phosphate. Further portions were treated with CoA at intervals, as indicated by the different symbols on the broken line on each graph. Each re-greened sample was also examined again periodically and the time course of A_{710} for each sample is shown by a continuous line with identical symbols along it. For further details see the text.

associated with loss of catalytic activity is again inconsistent with a chemical modification at the active site;

(iii) The failure of $(\text{NH}_4)_2\text{SO}_4$ precipitation or gel filtration of the de-greened enzyme to decrease re-greenability clearly shows that, whatever the nature of the factor responsible for the absorption band at 710nm it is not totally displaced from the protein by phenylmercuric acetate; this conclusion is also supported in part by the results of amino acid analysis (Engel & Massey, 1971b), showing that mercurial-treated enzyme retains its CoA even after extensive dialysis;

(iv) De-greening by phenylmercuric acetate is a slow process, taking about 30 min to approach completion. Release of the bound acyl-CoA, by contrast, cannot have a rate constant much smaller than 1 s^{-1} , since no lag is apparent in catalytic assays with a large excess of substrate (Engel & Massey, 1971a,b). The rate of de-greening is evidently not limited by the rate of release of bound thioester, though conceivably it could be limited by the infrequency of approach of the mercurial to a relatively inaccessible protein thiol group.

If a protein thiol group is not the site of the de-greening action of phenylmercuric acetate, what is the alternative? The experiments with enzyme samples de-greened by 4–5 mol of mercurial/mol showed unstable re-greening at 580nm by acetoacetyl-CoA, providing a reminder that mercurials can also react with thioesters. Normally such reactions release free acid from the thioester and would therefore not be reversed by the addition of an excess of thiol.

Another set of experiments, however, provides a possible explanation for this puzzling set of observations. Engel & Massey (1971b) showed that an acid extract prepared from green butyryl-CoA dehydrogenase contained all the CoA, and after neutralization could re-green the yellow form of the enzyme. Engel (1972) found that the instantaneous re-greening capacity of such protein-free extracts declined with time, and that the decline was partly reversed by prolonged incubation with yellow enzyme at pH 7 and room temperature. The parallel with the partly reversible decline in re-greenability of the enzyme after treatment with phenylmercuric acetate (Fig. 4) is striking. In both cases acid conditions appear to promote the decline, and alkaline conditions expedite recovery. This strongly suggests that the decline in re-greenability shown in Fig. 4 represents a change or changes in the bound small molecule rather than changes in the protein itself.

To explain the atypical instability of a thioester under mildly acid conditions, Engel (1972) proposed that there might be a cyclization of an oxoacyl-CoA to form a lactone and free CoA. An analogous

process has been described for the formation of triacetic lactone under certain conditions by the fatty acid synthetase complex of pigeon liver (Nixon *et al.*, 1968). If, even on the surface of the enzyme and in neutral buffer the postulated acyl-CoA compound exists in equilibrium with CoA and lactone, an excess of mercurial will react with the CoA and pull over the equilibrium. The phenylmercury-CoA mercaptide would very likely remain tightly bound, since the CoA moiety contributes much of the binding affinity of acyl-CoA ligands (Engel & Massey, 1971b). Since, however, only certain acyl-CoA compounds with unsaturated character in the acyl chain give long-wavelength absorption bands, the characteristic green colour of the native enzyme would be lost. The reactive lactone, in the absence of CoA with which to recombine, may perhaps react reversibly instead with protein side chains, possibly amino groups.

In this situation, the fragments of the original ligand might well be retained by the protein even through gel filtration or $(\text{NH}_4)_2\text{SO}_4$ precipitation. On the other hand, the non-covalently bound phenylmercury-CoA mercaptide at the active site would presumably be displaced by excess of substrate in a catalytic assay in exactly the same way as the ligand in the native green enzyme is displaced in such assays to allow full catalytic activity (Engel & Massey, 1971b). Re-greening would involve release of free CoA from the mercaptide and its recombination with the lactone. The speed of recovery at pH 8, as compared with pH 7 or 6, would be consistent with the addition of a thiolate anion to a lactone.

If the de-greening reaction indeed involves intermediate release of CoA from its thioester linkage, then this must occur on the surface of the enzyme. If it occurred in free solution, iodoacetate and iodoacetamide would be expected to de-green the enzyme. It is perhaps relevant that Steyn-Parvé & Beinert (1958a) found in their studies of the binding of acyl-CoA compounds to acyl-CoA dehydrogenases from pig liver that in the enzyme-substrate complex the thioester bond is acid-labile.

It cannot be too strongly emphasized that the properties of butyryl-CoA dehydrogenase discussed in the present paper are not only of interest in the context of microbial metabolism. Closely similar green complexes are also formed by the butyryl-CoA dehydrogenases of mammals. As pointed out previously (Engel & Massey, 1971b), an inhibitor that binds so tightly to an enzyme compulsorily involved in one of the two main energy-yielding metabolic pathways of mammalian cells is likely to be of regulatory significance. There is some evidence to suggest that the acyl-CoA dehydrogenase step may be rate-limiting in β -oxidation (Bunyan & Greenbaum, 1965; Stanley & Tubbs, 1974). It has also

been shown that inhibition of butyryl-CoA dehydrogenase by metabolites of hypoglycin A causes a major derangement of fatty acid and carbohydrate metabolism, which manifests itself clinically as Jamaican vomiting sickness (Osmundsen & Sherratt, 1975; Kean, 1976). In this context the identification of the tightly bound inhibitor in the native green form of the enzyme takes on added significance. Identification has been hampered in the past by an inadequate appreciation of the unusual chemical properties of this thioester. If the present interpretation is correct, it will perhaps provide a sounder basis for future experiment.

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