The effect on butyryl-CoA dehydrogenase of reagents specific for nucleophilic sulphur

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Removal of the green colour of butyryl-CoA dehydrogenase by reagents specific for nucleophilic sulphur is shown to involve chemical modification of the tightly bound CoA persulphide. 5,5'-Dithiobis-(2-nitrobenzoic acid) (Ellman's reagent) de-greens the enzyme essentially irreversibly, with a stoichiometry of approx. 1 mol/mol of FAD. A compound separated by subsequent gel filtration is eluted at the same position as a CoA-thionitrobenzoate standard. The ³⁵S-labelled distal sulphur atom of CoA persulphide is separated from this material. The enzyme remains fully active. Phenylmercuric acetate also de-greens the enzyme. The extent to which thiols restore the green colour declines with time. Gel filtration of mercurial-treated enzyme separates low-*M_r* material containing a CoA moiety, an extra S atom and a phenylmercury moiety. This material, added to yellow butyryl-CoA dehydrogenase with an excess of thiol, re-forms green enzyme, but it loses this ability on storage. The results are explicable if it is assumed that the thionitrobenzoate derivative of CoA persulphide loses the extra sulphur atom much more readily than does the phenylmercury derivative.

Butyryl-CoA dehydrogenase (EC 1.3.99.2) from *Megasphaera elsdenii* catalyses the reduction of a *trans*-double bond between C-2 and C-3 of short-chain acyl-CoA substrates *in vivo* (Baldwin & Milligan, 1964). This flavoprotein, like its counterparts involved in mammalian β -oxidation (Mahler, 1954; Steyn-Parvé & Beinert, 1958; Hoskins, 1966), is usually isolated in a green form with a broad absorption band centred at 710 nm (Engel & Massey, 1971a).

Palmer & Massey (1968) postulated that the green colour might arise from a charge-transfer interaction between enzyme-bound FAD and the negatively charged sulphur atom of a cysteine thiolate. Engel & Massey (1971a) reported de-greening by phenylmercuric acetate and Nbs₂, both usually specific for cysteine residues (Ellman, 1959; Webb, 1966). Several observations were, however, difficult to reconcile with the view that these reagents were modifying an active-site cysteine residue: (i) enzyme treated with phenylmercuric acetate was fully active (Engel & Massey, 1971a); (ii) the treated enzyme also formed a normal complex (Engel & Jones, 1978) with the inhibitor acetoacetyl-CoA; (iii)

although mercurial-treated enzyme could be 're-greened' (i.e. regain its absorption at 710 nm) on addition of a thiol reagent, such as dithiothreitol, this 're-greenability' declined with time; this decline was not, however, accompanied by any loss in catalytic activity (Engel & Massey, 1971a).

Reviewing the evidence, Engel & Jones (1978) concluded that the abolition of the absorption band at 710 nm by phenylmercuric acetate was due to chemical modification, not of the enzyme protein, but rather of a tightly bound CoA thioester, then thought to be responsible for the green colour (Engel & Massey, 1971b). If de-greening were caused by a cleavage of the thioester by the mercurial (Cecil & McPhee, 1959), reversal would require re-formation of the thioester bond. Since thioester cleavage is normally irreversible (Cecil & McPhee, 1959), it was proposed (Engel & Jones, 1978) that the greening ligand might be the CoA derivative of a keto acid or hydroxy acid, capable of cyclizing to form a lactone.

Recently it has been shown (Williamson *et al.*, 1982a,b) that the tightly bound CoA derivative is not a thioester but a persulphide, CoA-S-S⁻. The identification of this ligand has explained many previously puzzling experimental observations. In the present paper we examine the reaction of butyryl-CoA dehydrogenase with thiol-specific re-

Abbreviation used: Nbs₂, 5,5'-dithiobis-(2-nitrobenzoic acid) (Ellman's reagent).

agents and attempt to account for the experimental results in the light of the new information on the chemical nature of the green complex.

Materials and methods

Enzymes

Butyryl-CoA dehydrogenase was prepared by the method of Engel (1981) from *Megasphaera elsdenii*. The concentration of bound flavin was determined spectrophotometrically by using the following absorption coefficients: yellow butyryl-CoA dehydrogenase, $12.5 \text{ mm}^{-1} \cdot \text{cm}^{-1}$ at 450 nm (Engel & Massey, 1971a); green butyryl-CoA dehydrogenase, $10.4 \text{ mm}^{-1} \cdot \text{cm}^{-1}$ at 430 nm (Engel, 1981). Spectra were recorded with a Cary 219 scanning spectrophotometer. Experiments were performed in 0.1 M-potassium phosphate buffer, pH 7, at 25°C.

Radioactively labelled CoA persulphide was made and incubated with butyryl-CoA dehydrogenase as described by Williamson *et al.* (1982a). Unbound persulphide was removed by gel filtration. Scintillation counting was performed as described by Williamson *et al.* (1982a).

The bound ligand was removed from some enzyme samples by using a column of 3-(pyrid-2-ylthio)-2-hydroxypropyl ether Sepharose (from Pharmacia Fine Chemicals, Hounslow, Middx., U.K.) (Williamson & Engel, 1982). Enzyme thus treated is referred to as 'CoA-free' and is yellow.

The stoichiometry of compounds with respect to enzyme concentration is expressed per mol of enzyme-bound FAD, i.e. per mol of butyryl-CoA dehydrogenase subunits (Engel & Massey, 1971a).

Expression of 'greenness'

The 'greenness' of an enzyme sample is expressed either (i) as the A_{710}/A_{430} ratio or (ii) as a percentage of the maximum greenness (based on the A_{710}/A_{430} ratio). The highest A_{710}/A_{430} ratio theoretically obtainable has been estimated to be 0.54 (Engel & Massey, 1971a; Williamson *et al.*, 1982a). A sample with $A_{710}/A_{430} = 0.27$ is therefore designated as 50% green; 100% green butyryl-CoA dehydrogenase (with $A_{710}/A_{430} = 0.54$) contains 1 mol of bound CoA persulphide/mol of enzyme subunit (Williamson *et al.*, 1982a).

Reagents

Nbs₂, phenylmercuric acetate and dithiothreitol were purchased from BDH Chemicals, Atherstone, Warwicks., U.K. In the chemical-modification experiments, either a 4-fold molar excess of Nbs₂, compared with the concentration of enzyme subunits, or a 5-fold molar excess of phenylmercuric acetate was used, except where otherwise stated. Dithiothreitol (Cleland, 1964), when added, was always at a final concentration of 2 mM.

CoA was purchased from Sigma Chemical Co., Poole, Dorset, U.K. Acetoacetyl-CoA was prepared from CoA and freshly distilled diketene (Simon & Shemin, 1953; Lynen & Wieland, 1955).

Assay of enzyme activity

The assay of butyryl-CoA dehydrogenase activity was performed as described by Engel (1981), except that phenazine methosulphate was replaced by phenazine ethosulphate (Ghosh & Quayle, 1979).

Calibrated Sephadex G-25 column

A 20 cm × 1.5 cm column of Sephadex G-25 (Pharmacia Fine Chemicals) was equilibrated with 0.1 M-potassium phosphate buffer, pH 7, before separate application of standards (Table 1).

Absorption coefficient for CoA-thionitrobenzoate

A sample of CoA-thionitrobenzoate was made as described in the legend to Table 1, and gel-filtered. Its exact concentration was determined from the quantity of thionitrobenzoate anion liberated by dithiothreitol (Butterworth *et al.*, 1967). This was correlated with the original A_{260} to give $\epsilon_{260} = 17.5 \text{ mm}^{-1} \cdot \text{cm}^{-1}$.

Results

De-greening of butyryl-CoA dehydrogenase by Nbs₂

A sample of 42 μM-butyryl-CoA dehydrogenase ($A_{710}/A_{430} = 0.37$; 69% green) was incubated with a 4-fold excess of Nbs₂. The A_{710} declined, owing to 'de-greening' of the enzyme, and A_{412} increased, owing to release of thionitrobenzoate (Fig. 1a). Fig. 1(b), a plot of the change in A_{710}/A_{430} against thionitrobenzoate released, shows three distinct phases. (a) A small amount of thionitrobenzoate (0.12 mol/mol of enzyme) was released in the first few minutes without any decrease in A_{710} . (b) The next phase showed a decrease in A_{710}/A_{430} of 0.18, together with a release of 1.13 mol of thionitrobenzoate/mol of butyryl-CoA dehydrogenase. This decrease in A_{710}/A_{430} is equivalent to the conversion of 33% of the enzyme from the green form into the yellow form. The stoichiometry for this section of the curve is therefore 3.42 mol of thionitrobenzoate released/mol of enzyme de-greened. (c) In the final phase, a decrease in A_{710}/A_{430} of 0.12 was accompanied by the release of 0.21 mol of thionitrobenzoate/mol of enzyme, giving a stoichiometry of 0.95 mol of thionitrobenzoate released/mol of green butyryl-CoA dehydrogenase converted. The overall stoichiometry was 1.46 mol of thionitrobenzoate released/mol of butyryl-CoA dehydrogenase.

Free thionitrobenzoate was removed from Nbs₂-treated enzyme by gel filtration. The eluted flavoprotein showed no absorption band at 710 nm (Fig. 2).

Fig. 1. Spectral changes during reaction of Nbs_2 with green butyryl-CoA dehydrogenase

(a) A sample of $42 \mu\text{M}$ -butyryl-CoA dehydrogenase ($A_{710}/A_{430} = 0.37$) was incubated with $168 \mu\text{M}$ - Nbs_2 in 0.1 M -potassium phosphate buffer, pH 7, at room temperature. At suitable intervals, A_{710} ($\square$) and A_{412} ($\circ$) were recorded. (b) Amounts of thionitrobenzoate released, calculated from changes in A_{412} in (a), are plotted against the corresponding change in A_{710} divided by the original A_{430} which is an expression of the enzyme concentration. For this calculation ΔA_{412} is corrected by 5.3% for the small increase in the flavoprotein absorption at this wavelength.

The flavin absorption maximum in the visible region was shifted from 430 nm in the green enzyme to 450 nm in the Nbs_2 -treated enzyme. The flavin peak in the near-u.v. region was obscured by a new absorbance band at about 340 nm. Assay of the Nbs_2 -treated enzyme, however, showed no decrease in catalytic activity. The spectrum of the normal yellow form of the enzyme, without the band at 340 nm, was obtained by treating Nbs_2 -modified enzyme with dithiothreitol, and dialysing (Fig. 2). The difference spectrum (Fig. 2, inset) has a maximum at 330 nm, characteristic of mixed disulphides between aliphatic thiols and thionitrobenzoate (Ellman, 1959; Colman, 1969; Habeeb, 1972).

The usual reaction of Nbs_2 with an enzyme is the modification of cysteine thiol groups, often with a parallel loss of activity (e.g. Colman, 1969; Butterworth *et al.*, 1967; Olson & Massey, 1980).

Fig. 2. Spectra of Nbs_2 -modified and unmodified butyryl-CoA dehydrogenase

Green butyryl-CoA dehydrogenase was incubated with a 4-fold molar excess of Nbs_2 and gel-filtered. The enzyme obtained (—) was incubated with 2 mM -dithiothreitol and then dialysed against two changes of 0.1 M -potassium phosphate buffer, pH 7, before the spectrum was read again (---). The inset shows the difference between the two spectra.

However, butyryl-CoA dehydrogenase, degreened by Nbs_2 , is fully active. It seems improbable that modification of a protein group essential for formation of the green complex would not also influence catalytic activity. Nbs_2 therefore most probably reacts with the CoA persulphide ligand. The stoichiometry calculated from the final phase in Fig. 1(b) suggests that 1 mol of Nbs_2 reacts with 1 mol of CoA persulphide. The first two phases in Fig. 1(b) imply the modification of another, faster-reacting, group on the enzyme surface, such as an exposed thiol group, required for neither activity nor greening.

Analysis of Nbs_2 -modified enzyme on the calibrated Sephadex G-25 column

A sample of $173 \mu\text{M}$ -butyryl-CoA dehydrogenase ($A_{710}/A_{430} = 0.48$; 89% green) was incubated at pH 7 with a 4-fold molar excess of Nbs_2 . The A_{710}/A_{430} ratio declined to 0.07 (13% green) over 90 min (loss of 0.76 mol of green butyryl-CoA dehydrogenase/mol of total butyryl-CoA dehydrogenase). The increase in A_{412} indicated release of 1.14 mol of thionitrobenzoate/mol of enzyme. The elution profile on gel filtration (Fig. 3) shows an A_{340} peak of unchanged Nbs_2 at 53 ml. The A_{412} shows two peaks: the flavoprotein at 14 ml, and thionitrobenzoate at 40 ml. There are four peaks of 260 nm-absorbing material: protein (14 ml), thionitrobenzoate (40 ml), Nbs_2 (53 ml) and a peak at 29.5 ml. This last-mentioned peak, coincident with a

Fig. 3. Elution profile of Nbs_2 -treated butyryl-CoA dehydrogenase

A sample of $173 \mu\text{M}$ -butyryl-CoA dehydrogenase ($A_{710}/A_{430} = 0.48$) was incubated with $692 \mu\text{M}$ - Nbs_2 . When the A_{710}/A_{430} had decreased to 0.07, the enzyme was gel-filtered on the calibrated Sephadex G-25 column. Fractions were collected, and absorbance measurements were made at 260 nm ($\bullet$), 412 nm ($\square$) and 340 nm ($\circ$). The arrows indicate the elution positions of the following calibration standards: A, protein; B, CoA disulphide; C, CoA and S^{2-} ; D, CoA-thionitrobenzoate mixed disulphide; E, thionitrobenzoate anion; F, Nbs_2 .

pronounced shoulder in the main A_{340} peak, corresponds to the elution position of CoA-thionitrobenzoate. Furthermore, the A_{340}/A_{260} ratio of the fractions around 30 ml (about 0.45) is fairly close to that of the CoA-thionitrobenzoate standard ($A_{340}/A_{260} = 0.52$). Integration under this A_{260} peak indicates about 0.74 mol of CoA-thionitrobenzoate released/mol of enzyme loaded ($\epsilon_{260} = 17.5 \text{ mM}^{-1} \cdot \text{cm}^{-1}$).

The most-concentrated three fractions from the enzyme peak were pooled, and portions were treated as follows. (i) Incubation with dithiothreitol released 0.47 mol of thionitrobenzoate/mol of enzyme, as judged by A_{412} , without any change in A_{710} . After dialysis, the enzyme showed a spectrum identical with that of yellow butyryl-CoA dehydrogenase, as in the experiment shown in Fig. 2. (ii) Addition of a 6-fold molar excess of acetoacetyl-CoA led to formation of the enzyme-acetoacetyl-CoA complex with its characteristic absorbance band at 580 nm ($A_{580}/A_{430} = 0.35$). The increased absorption in the 320–340 nm region was still apparent. Addition of dithiothreitol, without prior dialysis or gel filtration of the enzyme, abolished this 'extra' absorbance and released 0.46 mol of thionitrobenzoate/mol of butyryl-CoA dehydrogenase, in reasonable agreement with the value from (i) above. (iii) Acetoacetyl-CoA was added as in (ii). The sample was then gel-filtered. The resultant enzyme ($A_{580}/A_{430} = 0.36$), on addition of dithiothreitol, released 0.33 mol

of thionitrobenzoate/mol of butyryl-CoA dehydrogenase, indicating that the acetoacetyl-CoA had displaced only 0.13 mol [cf. (ii)] of CoA-thionitrobenzoate from the enzyme. This implies that 0.33 mol of thionitrobenzoate/mol of butyryl-CoA dehydrogenase was covalently attached via cysteine residues. This value for protein-thionitrobenzoate mixed disulphide accounts for the difference between the amount of Nbs_2 that reacted with the enzyme (1.14 mol/mol) and the extent of de-greening (0.78 mol/mol). Since acetoacetyl-CoA displaced only 0.13 mol of CoA-thionitrobenzoate/mol of butyryl-CoA dehydrogenase, it can be calculated that 0.65 mol of CoA-thionitrobenzoate/mol of enzyme was released from Nbs_2 -treated enzyme after the first gel filtration, in reasonable agreement with the value of 0.74 mol of CoA-thionitrobenzoate/mol of enzyme obtained from Fig. 3 by integration.

Fate of the persulphide sulphur

The production and partial release of CoA-thionitrobenzoate does not by itself account for the cyanolysable sulphur (Williamson *et al.*, 1982a) originally associated with the CoA persulphide in the green form of the enzyme. The fate of this 'extra' sulphur was established in two ways. (i) A sample of $42 \mu\text{M}$ -butyryl-CoA dehydrogenase ($A_{710}/A_{430} = 0.37$; 69% green) containing CoA-S- $^{35}\text{S}^-$ was incubated with $168 \mu\text{M}$ - Nbs_2 at pH 7. When de-greening was only about 50% complete ($A_{710}/A_{430} = 0.22$; 41% green), dithiothreitol was added. The A_{710}/A_{430} ratio showed a very small increase to 0.24 (44% green). This simple experiment suggests that, on Nbs_2 modification of green butyryl-CoA dehydrogenase, at least one of the components of CoA persulphide (i.e. CoA or the persulphide sulphur) is irreversibly 'lost'. (ii) An identical sample of ^{35}S -labelled enzyme was treated with Nbs_2 as in (i). When A_{710}/A_{430} had declined to 0.11 (20% green), the sample was gel-filtered on the calibrated Sephadex G-25 column. A small amount of ^{35}S -labelled sulphur, 0.06 mol/mol of enzyme loaded, was eluted under a peak centred at 32 ml and therefore appears to be associated with CoA-thionitrobenzoate (cf. Table 1). A much larger amount of ^{35}S -labelled sulphur, 0.27 mol/mol of enzyme, was eluted under an unknown peak centred at 63 ml. This late peak may be an S^0 species, although attempts to run colloidal sulphur as a standard on Sephadex G-25 failed because the relatively large particles could not penetrate the gel. The remaining ^{35}S was eluted under the protein peak. Although the ^{35}S peaks at 32 and 63 ml account for only 0.33 mol of ^{35}S /mol of enzyme, as compared with 0.48 mol/mol for the de-greened enzyme, some released sulphur may re-bind to butyryl-CoA dehydrogenase (e.g. cysteine residues) (Williamson *et al.*, 1982a).

Reaction of the butyryl-CoA dehydrogenase-acetoacetyl-CoA complex with Nbs₂

Excess of acetoacetyl-CoA was added to yellow butyryl-CoA dehydrogenase, and the mixture was dialysed for 16 h at pH 7. The resulting enzyme sample (30 μ M), with $A_{580}/A_{430} = 0.36$, was incubated with 120 μ M-Nbs₂. The total increase in A_{412} , complete after 120 min, indicated release of 0.64 mol of thionitrobenzoate/mol of enzyme, with only a slight decrease in the A_{580}/A_{430} ratio to 0.35, and no loss in catalytic activity. After gel filtration, incubation of this enzyme sample with dithiothreitol gave a slow increase in A_{412} . The final A_{412} indicated 0.63 mol of thionitrobenzoate released/mol of butyryl-CoA dehydrogenase.

Evidently, bound acetoacetyl-CoA is not modified by Nbs₂, and no thionitrobenzoate mixed disulphides are lost on gel filtration. Furthermore, the enzyme-acetoacetyl-CoA complex, like the green form, still has 'non-essential' Nbs₂-reactive cysteine residues exposed.

Reaction of CoA-free butyryl-CoA dehydrogenase with Nbs₂

A sample of 11 μ M CoA-free yellow butyryl-CoA dehydrogenase released 1.31 mol of thionitrobenzoate/mol of enzyme on incubation with 46 μ M-Nbs₂. Again there was no change in catalytic activity.

De-greening of butyryl-CoA dehydrogenase by phenylmercuric acetate

A sample of 162 μ M-butyryl-CoA dehydrogenase ($A_{710}/A_{430} = 0.375$; 70% green) in 0.1 M-potassium phosphate buffer, pH 7, was incubated with a 5-fold molar excess of phenylmercuric acetate. Over a period of 35 min, A_{710}/A_{430} declined to 0.03 (6% green), in keeping with earlier results (Engel & Massey, 1971a; Engel & Jones, 1978). A portion was then gel-filtered, and the fractions containing enzyme were pooled (A_{710}/A_{430} for pooled enzyme = 0.04; 7% green). However, addition of dithiothreitol to the remaining mercurial-treated enzyme, which had not been gel-filtered, increased A_{710}/A_{430} to 0.17 (31% green). In both cases dithiothreitol was added 90 min after the initial addition of the mercurial.

In this experiment, gel filtration decreased the 're-greenability' of the mercurial-treated enzyme. In contrast, Engel & Jones (1978), using Tris/acetate buffer, pH 7.5, found that 're-greenability' was almost unchanged by gel filtration. It therefore appears that in phosphate buffer, unlike Tris/acetate buffer, some material of low M_r is released into solution during de-greening by phenylmercuric acetate. To test this hypothesis, butyryl-CoA dehydrogenase was labelled with either [¹⁴C]CoA or ³⁵S.

Release of ³⁵S on treatment of green butyryl-CoA dehydrogenase with phenylmercuric acetate

Butyryl-CoA dehydrogenase containing CoA-S-³⁵S- ($A_{710}/A_{430} = 0.45$; 83% green) was de-greened in 0.1 M-potassium phosphate buffer, pH 7, with a 5-fold molar excess of phenylmercuric acetate. After 25 min, A_{710}/A_{430} had declined to 0.11 (20% green). After gel filtration, and addition of dithiothreitol, the enzyme showed an increase in A_{710}/A_{430} to 0.17 (31% green). Gel filtration also revealed two peaks of ³⁵S, one associated with the protein at 14 ml (0.45 mol/mol of enzyme loaded) and a broader peak centred at 38 ml (0.55 mol/mol of enzyme), which is also the elution position of the phenylmercury-CoA mercaptide standard (Table 1). The initial de-greening modified 0.63 mol of greening ligand/mol of enzyme, since A_{710}/A_{430} decreased from 0.45 to 0.11. However, the re-greenability was slightly higher after gel filtration, and the overall loss of greening ligand was 0.52 mol/mol of butyryl-CoA dehydrogenase. This value is accounted for by the 0.55 mol of ³⁵S released/mol of enzyme.

Fate of [¹⁴C]CoA on treatment of green butyryl-CoA dehydrogenase with phenylmercuric acetate

A similar experiment to that described above was performed on butyryl-CoA dehydrogenase containing [¹⁴C]CoA-S-S-. A sample of 121 μ M-butyryl-CoA dehydrogenase ($A_{710}/A_{430} = 0.48$, and with 0.98 mol of [¹⁴C]CoA/mol of butyryl-CoA dehydrogenase) was incubated with a 5-fold excess of phenylmercuric acetate. When A_{710}/A_{430} had declined to 0.15 (28% green), the modified enzyme was gel-filtered. Addition of dithiothreitol to enzyme fractions produced no increase in A_{710}/A_{430} . Two peaks of [¹⁴C]CoA were seen, one corresponding to the flavoprotein peak (0.48 mol of [¹⁴C]CoA/mol of enzyme), and the other centred at 40 ml (0.55 mol of [¹⁴C]CoA/mol), again presumably representing phenylmercury-CoA mercaptide. The de-greening in this case involved the modification of 0.61 mol of CoA persulphide, a decrease from 89% to 28% green. Thus most (90%) of the [¹⁴C]CoA component of the modified ligand is accounted for by the peak centred at 40 ml.

Effect of the released low-M_r species on unliganded butyryl-CoA dehydrogenase

A sample of 468 μ M-green butyryl-CoA dehydrogenase ($A_{710}/A_{430} = 0.42$; 77% green) was completely de-greened with a 5-fold molar excess of phenylmercuric acetate and gel-filtered. The pooled material that was eluted between 36 and 40 ml showed an absorbance peak at 260 nm, with $A_{260} = 0.3$, and was then used in the following experiments, which are summarized in Table 2. (i) In the first, 0.45 ml of the '38 ml species' was added to 0.55 ml of CoA-free butyryl-CoA dehydrogenase to give a final enzyme

Table 1. Calibration of Sephadex G-25 column with reactants and likely products of the protein modifications

Details of the column are given in the text. The following details of each sample loaded refer to the numbers in the final column of the Table. Note 1: 0.2 ml of 0.1 mM green butyryl-CoA dehydrogenase was applied. Note 2: CoA disulphide was made by incubation of equimolar CoA and 1,1'-azobis-(*NN*-dimethylformamide) (Aldrich Chemical Co., Gillingham, Dorset, U.K.) (Kosower *et al.*, 1976; Kosower & Kosower, 1978) at 4°C for 3 h; 1.5 ml of a 0.5 mM solution was applied. Note 3: 1 ml of 1 mM-CoA solution was applied. Note 4: 0.1 ml of 1 mM- Na_2^{35}S solution was applied. Note 5: at pH 8, 0.29 mM-CoA was incubated with 0.33 mM-Nbs₂ for 15 min; the CoA-thionitrobenzoate mixed disulphide thus formed (Butterworth *et al.*, 1967):

was separated from the other reaction product, the thionitrobenzoate anion, on the calibrated Sephadex column (see note 7); the CoA-thionitrobenzoate mixed disulphide, after gel filtration, had an A_{340}/A_{260} ratio of 0.52. Note 6: phenylmercuric acetate itself has a relatively low absorption coefficient at its λ_{max} (257 nm), so that, in experiments involving other u.v.-absorbing material (e.g. protein, CoA), the contribution of phenylmercuric acetate is completely overshadowed; 0.6 mM-CoA and 1.2 mM-phenylmercuric acetate were incubated together for 30 min; the resulting phenylmercury-CoA mercaptide:

showed an absorbance maximum at 260 nm, due almost entirely to the CoA moiety; 1 ml of a 1 mM solution was applied. Note 7: the thionitrobenzoate anion was made by incubating CoA and Nbs₂ as indicated in note 2. Note 8: 1 ml of a 10 mM-phenylmercuric acetate solution was applied. Note 9: 1 ml of a 1 mM-Nbs₂ solution was applied.

Standard	Method of detection	Elution position (ml)	Note
Native butyryl-CoA dehydrogenase	A_{430}	14	1
CoA disulphide	A_{260}	22	2
CoA	A_{260}	27	3
Na_2^{35}S	^{35}S	28	4
CoA-thionitrobenzoate	A_{340}, A_{260}	30	5
Phenylmercury-CoA mercaptide	A_{260}	38	6
Thionitrobenzoate anion	A_{412}	42	7
Phenylmercuric acetate	A_{257}	45	8
Nbs ₂	A_{340}	52	9

Table 2. Extent of re-greening of butyryl-CoA dehydrogenase with the '38 ml species'

A 702 nmol sample of green butyryl-CoA dehydrogenase ($A_{710}/A_{430} = 0.42$) was de-greened by 3.51 mM-phenylmercuric acetate. Gel filtration on the calibrated Sephadex G-25 column yielded an A_{260} peak at 38 ml. The fractions between 36 and 40 ml were pooled, and this '38 ml species' was used as a potential re-greening agent. In the test samples, the final concentration of butyryl-CoA dehydrogenase was always 9.3 μM . In experiment (v) this was enzyme de-greened and gel-filtered as above; in all the other experiments CoA-free enzyme prepared as described in the Materials and methods section was used. Potassium phosphate buffer, pH 7 (0.1 M), was added to give a final volume of 1.0 ml. Volumes of the '38 ml species' are given in the Table. Absorption measurements were made after addition of 1 mM-dithiothreitol except where indicated: 'before dithiothreitol'; 'the '38 ml species' was preincubated with the dithiothreitol in this experiment'; 'the '38 ml species' was stored at 4°C for 16 h before use.

Expt.	Volume of '38 ml species' added (μl)	A_{710}/A_{430} ratio
(i)	450	0.04 ^a
(ii)	450	0.22
(iii)	450 ^b	0
(iv)	—	0
	250	0.18
	450	0.22
	650	0.26
	950	0.35
(v)	450	0.22
(vi)	950 ^c	0.07

concentration of 9.3 μM . The A_{710}/A_{430} ratio increased slightly to 0.04 (7% green). (ii) When the enzyme sample from (i) was incubated with dithiothreitol, the A_{710}/A_{430} ratio increased to 0.22 (41% green). Thus the '38 ml species' alone can hardly re-green at all. Addition of a thiol, however, yields the unmodified greening ligand. (iii) The '38 ml species' was preincubated with dithiothreitol for 10 min at 25°C. On addition of this mixture to 9.3 μM CoA-free butyryl-CoA dehydrogenase, however, no increase in A_{710} was observed, in contrast with (ii). Clearly, the '38 ml species' must bind to butyryl-CoA dehydrogenase before the addition of a thiol if greening is to occur. This is in keeping with earlier evidence (Engel, 1972; Williamson *et al.*, 1982a) that the free greening ligand is extremely unstable. (iv) Various amounts of the '38 ml species' were added to CoA-free butyryl-CoA dehydrogenase. After incubation with dithiothreitol, the extent of the increase in A_{710} was roughly proportional to the amount of the '38 ml species' added. (v)

Experiment (ii) was repeated with the gel-filtered phenylmercuric acetate-de-greened enzyme, instead of the unliganded enzyme used above. The observed increase in A_{710}/A_{430} of 0.22 (41% green) was identical with that seen in experiment (ii). (vi) the '38 ml species' was stored for 16 h at 4°C. A 0.95 ml portion of this aged sample was then added to CoA-free butyryl-CoA dehydrogenase, which was then incubated with dithiothreitol. The A_{710}/A_{430} ratio increased only to 0.07 (13% green), compared

with the value of 0.35 (65% green) obtained in (iv) above. With time, therefore, the '38 ml species' loses its capacity to regenerate the greening ligand on addition of a thiol.

Re-greening by addition of Na_2S

A sample of 27 μM -butyryl-CoA dehydrogenase ($A_{710}/A_{430} = 0.49$; 91% green) was incubated with a 5-fold excess of phenylmercuric acetate until A_{710}/A_{430} reached 0.03 (6% green), and was then gel-filtered. Addition of dithiothreitol to a portion of this enzyme increased the A_{710}/A_{430} ratio to 0.12 (22% green). Incubation of another portion with 1 mM- Na_2S and a 3-fold molar excess of CoA effected a rise in A_{710}/A_{430} to 0.50 (93% green). Thus modification by phenylmercuric acetate does not in any way alter the inherent ability of the flavoprotein to form a normal CoA persulphide-enzyme charge-transfer complex.

Discussion

The reaction of a typical disulphide with a persulphide results in formation of a trisulphide (Friedman, 1973):

The mechanism of de-greening by Nbs_2 of butyryl-CoA dehydrogenase is therefore proposed to be as follows:

The stoichiometry of the reaction is shown by the final slope of the line in Fig. 1(b). When the reaction of protein thiol group is complete, about 1 mol of thionitrobenzoate is released/mol of CoA persulphide modified.

De-greening by Nbs_2 brings about the release of a species that is eluted at 30 ml, which corresponds to the CoA-thionitrobenzoate standard. Since only 6% of the persulphide sulphur ^{35}S is eluted in this position, the species at 30 ml is likely to be mostly CoA-thionitrobenzoate, mixed with only a small amount of thio-CoA-thionitrobenzoate trisulphide. The remaining persulphide sulphur eluted under the protein peak centred at 14 ml, and also under an unknown peak (possibly of an S^0 species) centred at 63 ml. Tentatively, a rearrangement of the thio-CoA-thionitrobenzoate trisulphide may be envisaged, analogous to the formation of cystine and S^0 from thiocystine (Koj, 1980):

Effect of dithiothreitol on green butyryl-CoA dehydrogenase

A 1000-fold molar excess of dithiothreitol was added to green butyryl-CoA dehydrogenase in 0.1 M-potassium phosphate buffer, pH 7. No change in A_{710}/A_{430} was observed.

This would account for the observed elution profile.

Bivalent mercury is known to react stoichiometrically with the thiol group. This reaction can be reversed by addition of a thiol (Riordan & Vallee, 1972; Webb, 1966). By analogy, therefore, the mechanism of de-greening by phenylmercuric acetate is proposed to be as follows:

The '38ml species', released from enzyme de-greened by phenylmercuric acetate, contained CoA, persulphide sulphur and phenylmercury. It is proposed that this compound is the phenylmercury-thio-CoA mercaptide (above). Fully consistent with this view is the production of a 710nm-absorption band when this fraction was added back to butyryl-CoA dehydrogenase and incubated with a thiol.

The most striking difference between the reactions of Nbs₂ and phenylmercuric acetate with butyryl-CoA dehydrogenase is in the subsequent 're-greenability'. The A₇₁₀ of enzyme de-greened with mercurial could be partially recovered by addition of a thiol (Engel & Massey, 1971a; Engel & Jones, 1978), whereas Nbs₂-de-greened butyryl-CoA dehydrogenase could not be re-greened with thiols. This observation can be rationalized by assuming that the thio-CoA-thionitrobenzoate trisulphide is very labile, is weakly bound and rapidly breaks down with the irreversible loss of the persulphide sulphur. The phenylmercury-thiol-CoA mercaptide is also weakly bound, but is more stable. The isolated mercaptide only slowly loses its ability to re-green butyryl-CoA dehydrogenase over a period of hours. Mercury salts of CoA itself are insoluble (Jaenicke & Lynen, 1960), and this decay could conceivably be due to slow precipitation. Alternatively, there may be an expulsion of S⁰ from the phenylmercury-thio-CoA mercaptide, analogous to that postulated above for the decay of the thio-CoA-thionitrobenzoate trisulphide.

The results described in the present paper appear to resolve the remaining uncertainties (Engel & Jones, 1978) with regard to the de-greening of butyryl-CoA dehydrogenase by thiol-specific reagents. There seems to be little doubt that de-greening is due to the attack of these reagents on bound CoA persulphide. The irreversibility of this process in the case of Nbs₂, and declining reversibility in the case of the mercurial, reflect the instability of the derivatives that result.

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References

- Baldwin, R. L. & Milligan, L. P. (1964) *Biochim. Biophys. Acta* **92**, 421–432
- Butterworth, P. H. W., Baum, H. & Porter, J. W. (1967) *Arch. Biochem. Biophys.* **118**, 716–723
- Cecil, R. & McPhee, J. R. (1959) *Adv. Protein Chem.* **14**, 255–389
- Cleland, W. W. (1964) *Biochemistry* **3**, 480–482
- Colman, R. F. (1969) *Biochemistry* **8**, 888–898
- Ellman, G. L. (1959) *Arch. Biochem. Biophys.* **82**, 70–77
- Engel, P. C. (1972) *Z. Naturforsch. Teil B* **27**, 1080–1081
- Engel, P. C. (1981) *Methods Enzymol.* **71**, 359–366
- Engel, P. C. & Jones, J. B. (1978) *Biochem. J.* **171**, 51–59
- Engel, P. C. & Massey, V. (1971a) *Biochem. J.* **125**, 879–887
- Engel, P. C. & Massey, V. (1971b) *Biochem. J.* **125**, 889–902
- Friedman, M. (1973) *The Chemistry and Biochemistry of the Sulphydryl Group in Amino Acids, Peptides and Proteins*, pp. 199–229, Pergamon Press, Oxford
- Ghosh, R. & Quayle, J. R. (1979) *Anal. Biochem.* **99**, 112–117
- Habeeb, A. F. S. A. (1972) *Methods Enzymol.* **25**, 457–464
- Hoskins, D. D. (1966) *J. Biol. Chem.* **241**, 4472–4479
- Jaenicke, L. & Lynen, F. (1960) *Enzymes 2nd Ed.* **3**, 3–103
- Koj, A. (1980) in *Natural Sulphur Compounds* (Cavallini, D., Gaull, G. E. & Zappia, V., eds.), pp. 463–470, Plenum Press, New York and London
- Kosower, N. S. & Kosower, E. M. (1978) *Int. Rev. Cytol.* **54**, 109–160
- Kosower, N. S., Kosower, E. M. & Wertheim, B. (1976) *Biochem. Biophys. Res. Commun.* **37**, 593–596
- Lynen, F. & Wieland, O. (1955) *Methods Enzymol.* **1**, 566–573
- Mahler, H. R. (1954) *J. Biol. Chem.* **206**, 13–26
- Olson, S. T. & Massey, V. (1980) *Biochemistry* **19**, 3137–3144
- Palmer, G. & Massey, V. (1968) in *Biological Oxidations* (Singer, T. P., ed.), pp. 263–300, Interscience, New York
- Riordan, J. F. & Vallee, B. L. (1972) *Methods Enzymol.* **25**, 449–456
- Simon, E. J. & Shemin, D. (1953) *J. Am. Chem. Soc.* **75**, 2520
- Steyn-Parvé, E. P. & Beinert, H. (1958) *J. Biol. Chem.* **223**, 843–852
- Webb, J. L. (1966) *Enzyme and Metabolic Inhibitors*, vol. 2, pp. 729–985, Academic Press, New York
- Williamson, G. & Engel, P. C. (1982) *Biochem. Biophys. Acta* **706**, 245–248
- Williamson, G., Engel, P. C., Mizzer, J. F., Thorpe, C. & Massey, V. (1982a) *J. Biol. Chem.* **257**, 4314–4320
- Williamson, G., Engel, P. C., Nishina, Y. & Shiga, K. (1982b) *FEBS Lett.* **138**, 29–32