The Purification and Properties of Butyryl-Coenzyme A Dehydrogenase from *Peptostreptococcus elsdenii*

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Butyryl-CoA dehydrogenase prepared by a simple procedure from *Peptostreptococcus elsdenii* has a molecular weight of approx. 150 000. The enzyme has FAD as its prosthetic group. The amino acid analysis is reported. This enzyme, like most of the corresponding mammalian ones, is green. The absorption band at 710 nm can be abolished irreversibly by dithionite reduction and air reoxidation; it can be abolished reversibly by phenylmercuric acetate or potassium bromide. The enzyme as isolated appears to be a mixture of a green and a yellow form, both of which are active. This view is supported by the variable 'greenness' of different preparations and the biphasic curve obtained in anaerobic spectrophotometric titrations with dithionite. It can be calculated from the titration results that fully green enzyme would have a peak-to-peak absorption ratio (E_{710}/E_{430}) as great as 0.54. The green form is much less rapidly reduced by dithionite than the yellow form, but is nevertheless much more readily reduced by dithionite than the enzyme from pig liver. It is also more readily reoxidized by air and shows less tendency to form a semiquinone. Treatment with sodium borohydride produces an unusual reduced species that is probably the 3,4-dihydroflavin.

Peptostreptococcus elsdenii, a Gram-negative anaerobe first isolated from sheep rumen (Elsden, Gilchrist, Lewis & Volcani, 1951; Elsden, Volcani, Gilchrist & Lewis, 1956) ferments glucose, lactate and various other simple carbon compounds. It surmounts the problem of excess of reducing power by excreting short-chain fatty acids (Elsden & Lewis, 1953). The short-chain acyl-CoA dehydrogenase, a key enzyme in this metabolism, is present in large amounts. Baldwin & Milligan (1964) partially purified this enzyme and showed that, like the mammalian acyl-CoA dehydrogenases (Crane, Mii, Hauge, Green & Beinert, 1956; Crane & Beinert, 1956), it requires a flavoprotein electron acceptor/donor. Another feature shared with butyryl-CoA dehydrogenase from ox, pig and monkey liver (Mahler, 1954; Stéyn-Parvé & Beinert, 1958; Hoskins, 1966), though not sheep liver (Seubert & Lynen, 1953), is the bright green colour, caused by a broad absorption band centred at 710 nm. Such similarities are striking because the mammalian enzymes are primarily dehydrogenases whereas that from *P. elsdenii* is apparently a reductase.

In the present paper we describe a simple procedure for the purification of butyryl-CoA dehydrogenase from *P. elsdenii*. The procedure is not complicated by interference (Crane *et al.* 1956) from

similar enzymes of different chain-length specificity. The high yields make this enzyme ideal for the study of the unusual features of acyl-CoA dehydrogenases, and some of the properties of the enzyme are described below.

MATERIALS AND METHODS

Growth of cells. Low-iron cultures of *P. elsdenii* strain LCI were grown, harvested, dried, ground and stored as described by Mayhew & Massey (1969) except that commercial sodium lactate was used (Pfanstiehl Laboratories, Chicago, Ill., U.S.A.). Occasionally the cells were disrupted by sonication rather than drying and grinding. Although both procedures give equally good results, the drying method has the advantage that the powder may be stored at -15°C for many months without diminishing the final yield of enzyme, and small amounts can be prepared as required. It is essential, however, that the drying be thorough and rapid.

Purification of the enzyme. Powdered cells (20 g) were gradually suspended at room temperature in 200 ml of vigorously stirred water. After 5–6 h of stirring the suspension was centrifuged at 20 000g for 20 min. After decantation of the greenish supernatant the grey pellet was resuspended in about 60 ml of water and centrifuged again. The second, much paler, supernatant was added to the first, and the solution was dialysed for 12 h at 2°C , the temperature for all subsequent steps, against 0.2M-potassium phosphate buffer, pH 7. The cold dialysed solution was adjusted to pH 5.2 with 2M-acetic acid and the pale-grey precipitate was removed by centrifugation.

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After readjustment to pH 7 with 1M-KHCO₃, the solution was loaded on to DEAE-cellulose (Whatman DE 32), equilibrated with 0.2M-potassium phosphate buffer, pH 7, in a column (30 cm $\pm$ 3.5 cm) without constrictions at the top or bottom. The column usually showed, after loading, a narrow pale-grey band at the top, a broader bright-green band below it and a yellow band (flavodoxin), which was eluted on further washing with 0.2M-potassium phosphate, pH 7. The column was washed extensively with 0.25M-potassium phosphate, pH 7, which removed all traces of flavodoxin and widened the two remaining bands. If the phosphate concentration was raised further, either batchwise or with a gradient, the green material was eluted as a very dilute solution in a correspondingly large volume. Therefore the column was allowed to run just dry, and the DEAE-cellulose was then extruded intact on to a clean surface. The clearly defined green band was cut from the band above and the clean column material below, and was resuspended in water. The slurry was poured into a narrow (30 cm $\pm$ 2 cm) column with 2–3 mm of clean DEAE-cellulose at the bottom. This column was then treated with an eluent made by adjusting 0.6M-potassium phosphate, pH 7, to pH 6 with acetic acid and to 5% saturation with (NH₄)₂SO₄ (Enzyme Grade; Mann Research Laboratories, New York, N.Y., U.S.A.). The bright-green eluate was dialysed against 0.1M-potassium phosphate, pH 7, and then adjusted to 50% saturation with (NH₄)₂SO₄. A pale precipitate was discarded and the supernatant adjusted to 80% saturation. The solution was centrifuged after stirring for $\frac{1}{2}$ h. This gave a pale-yellow supernatant and a grass-green precipitate, which, on resuspension in a little 0.1M-potassium phosphate, pH 7, and further centrifugation, gave a clear dark-green solution. This was then dialysed against the same buffer. Further small amounts of impurity could be removed by chromatography on Sephadex G-200.

The purified enzyme represents 1–2% of the total dry wt. of the cells. Suspensions or solutions in concentrated (NH₄)₂SO₄ may be satisfactorily stored in the refrigerator or the deep-freeze. Suspensions in (NH₄)₂SO₄ may be freeze-dried after centrifugation without any alteration of the spectrum on redissolving.

For reasons discussed below, the absorption spectrum, although a useful indication, is not an infallible criterion of purity. The preparation was homogeneous as judged by electrophoresis on cellulose acetate strips, chromatographic behaviour and reproducibility of amino acid analysis. We nevertheless suspect contamination with small but significant amounts of at least one other protein, an enoyl-CoA hydrolase. This is discussed in the accompanying paper (Engel & Massey, 1971).

Assay of the enzyme. Assays for butyryl-CoA dehydrogenase are unsatisfactory for several reasons (Crane *et al.* 1956). If the natural electron acceptor ETF* (Crane & Beinert, 1956) is used to mediate reduction of cytochrome *c* or a dye, interpretation is complicated by the presence of unknown and variable amounts of ETF in the enzyme preparation. Also, such a procedure assumes that similar problems in the purification of the ETF to be used have been solved. If the dehydrogenase is directly coupled to a dye, ETF contamination remains a problem. The dye-

coupled reactions are non-specific: thiol groups, for instance, reduce 2,6-dichlorophenol-indophenol. In the initial stages of purification very active thiol esterases are present and a substrate-dependent reduction of 2,6-dichlorophenol-indophenol may be caused by free CoA released by their action.

Some of these problems have been diminished by using phenazine methosulphate to couple the dehydrogenase to 2,6-dichlorophenol-indophenol. Because the rate of reduction of 2,6-dichlorophenol-indophenol is greatly increased by the addition of phenazine methosulphate, smaller amounts of enzyme are required for assay and the interfering reactions become less significant. The conditions for this assay are as follows: 0.2M-sodium phosphate, pH 8, 0.001% 2,6-dichlorophenol-indophenol and 0.1 mM-butyryl-CoA [made by the method of Simon & Shemin (1953)] are mixed and warmed to 25°C; enzyme is added, followed by phenazine methosulphate (0.004% final concn.) added in dim light to prevent photoreactions. The decline in E_{600} is recorded. The 2,6-dichlorophenol-indophenol has an ϵ of 22000 l·mol⁻¹·cm⁻¹ at this wavelength (Armstrong, 1964). The purified enzyme has a specific activity of 200 μ mol/min per μ mol of enzyme-bound flavin in this assay.

This cumbersome assay was little used during the routine purification, because the distinctive spectral properties of the enzyme provide a more convenient guide to the purification.

Amino acid analysis. Analyses by the method of Spackman, Stein & Moore (1958) were very kindly done by Dr Severino Ronchi and Dr C. H. Williams, jun., with a Beckman model B Analyser. Samples were extensively dialysed against 1 μ M-potassium phosphate buffer, pH 7, then dried, and hydrolysed for 24 h in 6.27M-HCl. The hydrolysates were dried, then dissolved in 0.2M-sodium citrate buffer, pH 2.2, and applied to the column. For some analyses the acid hydrolysis was performed in the presence of added dimethyl sulphoxide, as described by Spencer & Wold (1969). Tryptophan was determined by the method of Spies & Chambers (1949) as modified by Harrison & Hofmann (1961).

Flavin analysis. The protein, in 0.1M-potassium phosphate buffer, pH 7, was treated with 0.33 vol. of cold 20% (w/v) trichloroacetic acid. The white pellet, after centrifugation, was washed three times with 1 vol. of 5% (w/v) trichloroacetic acid to ensure quantitative removal of the flavin. The combined supernatants were thoroughly extracted with ether, which was evaporated by a stream of N₂. The solution was made up to a known volume and its absorption spectrum was recorded.

A sample of the yellow extract was subjected to ascending chromatography on 20 cm precoated plastic t.l.c. sheets (MN Polygram SIL S-HR; Macherey, Nagel and Co., Düren, Germany) for 1–2 h. Commercial samples of FAD and FMN (Sigma Chemical Co., St Louis, Mo., U.S.A.) were used as standards.

Anaerobic titrations. The enzyme was titrated with sodium dithionite in the apparatus described by Foust, Burleigh, Mayhew, Williams & Massey (1969). The titrant was standardized by titrating lumiflavin 3-acetic acid. The anaerobic conditions were achieved by repeated evacuation followed by flushing with N₂ 'scrubbed' with Fieser's solution (Fieser, 1929).

Anaerobic EDTA-light reductions. These were done as

* Abbreviation: ETF, electron-transferring flavo-protein.

described by Massey & Palmer (1966), except that the light-source was a ring of six 15 W incandescent light-tubes.

Spectrophotometry. Absorption spectra were recorded with a Cary 14 scanning spectrophotometer. Catalytic assays were measured on a Gilford recording spectrophotometer.

RESULTS

Spectrum of the enzyme. A typical absorption spectrum shown in Figs. 1(a) (visible) and 1(b) (ultraviolet) displays maxima at 710, 430, 365 and 266nm, with extinctions in the proportion 0.43:1:0.78:7.3 and minima at 530, 386, 315 and 245nm. It is strikingly similar to the previously described spectra of green mammalian butyryl-CoA dehydrogenases (Mahler, 1954; Steyn-Parvé & Beinert, 1958; Hoskins, 1966). The long-wavelength band centred at 710nm is unusually pronounced; however, the E_{710}/E_{430} ratio (0.43) is considerably higher than the reported values. This ratio for Baldwin & Milligan's (1964) preparation from *P. elsdenii* was also high, but the absence of either a clear maximum at 710nm or a clear minimum at 530nm suggests that their preparation may have been less pure than ours.

The main peaks in the visible region are unresolved and are found at lower wavelengths than those of free flavins and most flavoproteins. The u.v. peak at 266nm is also at an unusually low wavelength even for a flavoprotein. The corresponding peak for the pig enzyme is at 266nm and that for the bovine enzyme is even lower, at 255nm.

It was noticed at an early stage of this work that, even with apparently homogeneous samples, the visible absorption spectrum was not entirely

constant from one preparation to another. Although for most preparations the E_{710}/E_{430} ratio was between 0.33 and 0.46, this ratio was occasionally as low as 0.06. The less-green preparations always showed a shift of the main flavin peak towards 450nm. A search for the source of this variability revealed that the greenness of the purified enzyme was related to the extent of oxidation of the crude extract. Many of the flavoprotein components are reduced by the abundant oxidizable substrates (unused lactate and short-chain fatty acids) in the crude extract. If such an extract is loaded on to a DEAE-cellulose column in 0.1M-potassium phosphate buffer, a dark-purple band, presumably semiquinoid flavoprotein, is bound near the top, and this gradually turns orange-yellow on washing with buffer. If the stirring at room temperature and the dialysis before chromatography are omitted from the purification procedure, the butyryl-CoA dehydrogenase so obtained has a low E_{710}/E_{430} ratio. The same result is achieved if the dialysis is performed anaerobically. These procedures do not affect the total yield of pure enzyme in terms of protein, flavin or catalytic activity. We have attempted in general to obtain enzyme with the maximum amount of green colour, but as discussed below, we have reason to believe that even our greenest preparations (E_{710}/E_{430} 0.46) are not fully green.

Identification of the prosthetic group. The yellow trichloroacetic acid extract showed absorption maxima at 373 and 448nm. Published peak positions are 373 and 445nm for FMN and 375 and 450nm for FAD (Whitby, 1953). The peak-to-peak ratios are 0.85 for FMN, 0.82 for FAD, and that

Fig. 1. Typical visible (a) and u.v. (b) spectra of butyryl-CoA dehydrogenase in 0.1M-potassium phosphate buffer, pH 7. These spectra were measured in silica cells with 1 cm light-path. In both cases the 0-1 slidewire of the Cary spectrophotometer was used. The sample used for (b) was a dilution of that in (a).

Table 1. *Thin-layer chromatography of the prosthetic group of butyryl-CoA dehydrogenase*

R_F values are given only for the main spots detected under u.v. light. The standards gave several minor spots in solvent systems 1, 3 and 4, and the unknown gave two minor spots in system 1 and one minor spot in system 3.

Solvent	R_F values		
	FAD	Unknown	FMN
1. 5% Na_2HPO_4	0.71	0.73	0.55
2. Formic acid-isopentanol-water (1:3:1, by vol.)	0.87	0.83	0.90
3. Propan-1-ol- NH_3 -water (6:3:1, by vol.)	0.43	0.38	0.21
4. Butan-1-ol-acetic acid-water (4:1:5, by vol.) (upper phase)	0.09	0.09	0.27

observed here was 0.824. On addition of a few crystals of *Naja naja* venom to a dilute solution of the extract the fluorescence increased by a factor of 10.7 (excitation at 450nm, emission at 540nm measured with an Aminco-Bowman fluorimeter). With FMN no rise is expected, whereas with FAD the phosphodiesterase treatment should result in a tenfold increase in fluorescence (Bessey, Lowry & Love, 1949). The results of t.l.c. of the extract are shown in Table 1. On the basis of these results it seems reasonable to conclude that the prosthetic group of butyryl-CoA dehydrogenase from *P. elsdennii* is FAD. The bovine enzyme also has FAD as its prosthetic group (Mahler, 1954). The ϵ of the bacterial enzyme at 430nm can be calculated to be $96001 \cdot \text{mol}^{-1} \cdot \text{cm}^{-1}$ based on an ϵ of $113001 \cdot \text{mol}^{-1} \cdot \text{cm}^{-1}$ for FAD at 450nm (Whitby, 1953).

Amino acid analysis. Table 2 gives the results of a 24h acid hydrolysis uncorrected for destruction or incomplete release of threonine, serine, valine, leucine and isoleucine. Enzyme solutions containing approx. 15nmol of bound flavin were used for each analysis. The analyses for tryptophan were done separately on heat-denatured holo-enzyme and acid-denatured apoenzyme (55nmol of each). The mean value obtained was 1.1 residues/mol of flavin. The results were correlated with a careful determination of flavin, by extraction with trichloroacetic acid, on the same sample, and indicate a minimum molecular weight of 43400, corresponding to 1mol of FAD. This compares with values of 30000 (Mahler, 1954) and 71000 (Steyn-Parvé & Beinert, 1958) for the mammalian enzymes, based on flavin extraction and biuret reaction.

Molecular weight. The approximate mol. wt. was estimated to be 150000 with a calibrated Sephadex G-150 column (Andrews, 1964). The results are shown in Fig. 2.

Reduction by dithionite. One of the striking features of pig liver butyryl-CoA dehydrogenase described by Steyn-Parvé & Beinert (1958) is the irreversible destruction of the 710nm absorption band on reduction with sodium dithionite and reoxidation by air. Fig. 3 shows that the same phenomenon occurs with the bacterial enzyme.

Table 2. *Amino acid composition of butyryl-CoA dehydrogenase from P. elsdennii*

Amino acid	Content (residues/mol of flavin)
Lysine	32
Histidine	5
Ammonia	35
Arginine	9
Aspartic acid	38
Threonine	28
Serine	14
Glutamic acid	48
Proline	10
Glycine	44
Alanine	44
$\frac{1}{2}$ -Cystine	5
Valine	26
Methionine	11
Isoleucine	25
Leucine	31
Tyrosine	14
Phenylalanine	18
Taurine	1.02*
β -Alanine	0.80*
Tryptophan	1
	440

* The analysis was largely undertaken with the object of ascertaining whether these two unusual amino acids were present. The significance of their presence is discussed in the accompanying paper (Engel & Massey, 1971).

However, the reduction by dithionite occurs much faster than with the mammalian enzyme and is complete within minutes rather than days. If the reduction is followed spectrophotometrically at 430 or 450nm, it is seen to be biphasic, 20–30% of the bleaching occurring instantaneously. Monitoring the change at 710nm reveals that the rapid bleaching does not alter the long-wavelength absorption, which is gradually abolished in parallel with the slow phase of bleaching at 430nm. This can also be seen by eye: immediately after addition of solid sodium dithionite the enzyme solution appears to be a much bluer green than before.

On reoxidation by air the spectrum becomes much more nearly that of a normal flavoprotein with a

Fig. 2. Estimation of molecular weight on a calibrated column of Sephadex G-150. The buffer used was 0.1M-sodium phosphate, pH7. The calibration proteins were ox heart cytochrome c (A, mol.wt. 12400), ribonuclease (B, mol.wt. 13700), soya-bean trypsin inhibitor (C, mol.wt. 21500), ovalbumin (D, mol.wt. 45000), bovine serum albumin (E, mol.wt. 67000, and G, mol.wt. 134000) and ox liver catalase (H, mol.wt. 248000), all commercial samples, and 'old yellow enzyme' from yeast (F, mol.wt. 101000) kindly provided by Dr Rowena G. Matthews. The elution volume of butyryl-CoA dehydrogenase is indicated by the open circle.

peak extinction at 450nm about 30% higher than the peak extinction at 430nm before reduction. Dialysis to remove sulphite and other oxidation products of dithionite does not restore the original spectrum, but sometimes removes a little flavin. Extraction of the FAD with trichloroacetic acid yields an ϵ of $125001 \cdot \text{mol}^{-1} \cdot \text{cm}^{-1}$ for this yellow form of the enzyme.

The cycle of reduction by dithionite and re-oxidation by air does not alter the specific activity in a catalytic assay. If, however, the same enzyme sample is subjected to a second cycle, the spectrum of the leuco form is produced instantaneously without any slow phase.

No semiquinone is detected during reduction, but when concentrated enzyme solutions are reoxidized by air a small amount of blue semiquinone (Massey & Palmer, 1966) is seen.

The green and yellow forms of the enzyme were titrated anaerobically with standard sodium dithionite solution as shown in Fig. 4. It was found that 1 mol of dithionite (2 electrons) is required per mol of flavin for the full reduction of the yellow enzyme. The titration of the green form, on the other hand, is more complex. A plot of E_{425}

Fig. 3. Reduction of green butyryl-CoA dehydrogenase by sodium dithionite. The reduction was done at room temperature in a stoppered 1 ml cuvette (1 cm light-path). The buffer was 0.1M-potassium phosphate, pH7. Excess of reductant was used, i.e. a few grains of the solid. The spectra shown are those of the initial green enzyme (—), the leucoprotein (----) and the yellow form obtained on readmission of air (-----).

against added reductant is linear and the end point indicates an average uptake of 2.76 electrons/mol of flavin. A similar plot of the E_{710} shows a discontinuity, however: in agreement with the kinetics of reduction by excess of dithionite described above, the first 20–25% of the dithionite required for full reduction does not affect the E_{710} .

These results suggest that the enzyme as isolated represents a mixture of a green and a yellow form. Different proportions of these forms would explain the variable spectra obtained with different purified samples. Recalculation on the basis of a 2-electron reduction of the yellow component shows that the green component takes up 2.97 electrons/mol of flavin. One can also estimate that for fully green enzyme the E_{710}/E_{430} ratio is likely to be about 0.54.

EDTA-light reduction. Reduction by illumination of an anaerobic solution of enzyme in 0.1M-potassium phosphate buffer, pH7, containing 0.1M-EDTA (Massey & Palmer, 1966) was undertaken to try to obtain larger yields of semiquinone. The reduction of green enzyme, like that with dithionite, was biphasic, and 20–25% of the reduction occurred rapidly. The slow phase took several days and there was no evidence of semiquinone production. Yellow enzyme could be reduced under the same conditions in about 1 h and there was a slight rise in the extinction between 500 and 600nm. The reduction of green enzyme could be accelerated by the addition of FAD, and, under these conditions, reoxidation by air yielded only the yellow form of the enzyme.

Fig. 4. Anaerobic spectrophotometric titration of butyryl-CoA dehydrogenase by sodium dithionite. In (a) 191 nmol (in terms of flavin) of the yellow form in 1.92 ml of 0.1 M-potassium phosphate, pH 7, was titrated with 3.49 mM-dithionite. The peak extinction at 450 nm is plotted. In (b) 133 nmol (in terms of flavin) of the green form in 2.46 ml of 0.17 M-potassium phosphate, pH 7.7, was titrated with 2.83 mM-dithionite. The titration is shown in terms of bleaching at 425 nm (●) and 710 nm (○). All points are corrected for dilution.

Reduction by sodium borohydride. Addition of excess of sodium borohydride to a solution of the green enzyme at pH 7 produced a marked change in the spectrum (Fig. 5). The peaks at 365 and 430 nm were replaced by a peak at 393 nm and two slightly smaller ones at 370 and 414 nm. The peak at 710 nm was decreased by about 25%. This solution displayed a strong pale-blue fluorescence under u.v. illumination. On reoxidation of the sample by air, the spectrum showed a slight but significant recovery of the extinction at 710 nm. In the region of the main flavin peaks the spectrum resembled the original spectrum although the extinction was somewhat decreased at 430 nm. When the solution was illuminated with incandescent light tubes the spectrum changed again, with the peak at 710 nm being gradually abolished, that at 430 nm being diminished and shifted to 445 nm, and a shoulder emerging at 475 nm.

When the enzyme in its yellow form, produced by dithionite reduction and reoxidation by air, was treated with borohydride, the spectrum showed a peak at 392 nm, much smaller than the initial 450 nm peak, and with shoulders at 375 and 410 nm. On aeration there was a rise in the extinction between 330 and 530 nm. The spectral features described above remained, but, in addition, a large extinction at 450 nm appeared. As with the green enzyme, the spectrum became like that of the original on exposure of the sample to room light at 25°C for 2 h.

These observations are very similar to the findings with D- and L-amino acid oxidases (Massey, Curti, Müller & Mayhew, 1968), indicating conversion of the flavin into the 3,4-dihydro form by borohydride.

Fig. 5. Effect of excess of sodium borohydride on green butyryl-CoA dehydrogenase. Solid was added to a solution of the enzyme in 0.1 M-potassium phosphate buffer, pH 7.0, at room temperature. The spectra shown are those of the initial green enzyme (-----), borohydride-reduced enzyme (—) and reoxidized enzyme (---).

Effect of thiol reagents. A titration with phenylmercuric acetate (microanalytical standard; Hopkin and Williams Ltd., Manchester, U.K.) showed that about 2 mol/mol of flavin could be added without permanently altering the visible spectrum of the green form of butyryl-CoA dehydrogenase. A further 2 mol, however, removed the long-wavelength absorption band and shifted the other visible peak to 450 nm with a concomitant rise in the extinction. These changes, shown as a difference spectrum in Fig. 6, are very similar to those observed on dithionite reduction and reoxidation by air. An additional similarity is the rapid reduction by dithionite of both these yellow forms of the enzyme.

When the E_{710} was measured with time after

addition of the first 1–2 mol of mercurial, a rapid decrease in the first few seconds was observed, followed by a slow rise to the initial value over the course of the next few minutes. This suggested that kinetically more accessible groups involved in the maintenance of the green complex were ultimately losing the mercurial to other groups, possibly revealed by partial unfolding of the protein.

Excess of dithiothreitol (Calbiochem, Los Angeles, Calif., U.S.A.) that was added soon after titration with phenylmercuric acetate restored about 90% of the E_{710} . This is the first reported reversible 'de-greening' of butyryl-CoA dehydrogenase. If, however, the enzyme was left for 24 h in ice before the dithiol was added, no long-wavelength extinction returned. The mercurial-treated enzyme was fully active in catalytic assays both after and before addition of dithiothreitol, even when left for 24 h before assay. Mercurial was present in the assay mixtures to exclude the possibility of dissociation on dilution.

When an untreated sample of green enzyme was added to an equal sample 'de-greened' with 4 mol of mercurial/mol of flavin, there was a rapid rise in E_{710} and the final visible spectrum was indistinguishable from that of the untreated sample. This was taken to indicate a direct intermolecular exchange of mercurial and it suggests that the chromophore is at the surface of the enzyme molecule. It is possible, however, that the mercurial first dissociated from the treated enzyme, and only secondarily reacted with the untreated enzyme.

In a further survey of thiol reagents, iodoacetic acid, iodoacetamide and arsenite were all found to

be without effect on the spectrum even when added in large excess. However, small molar excesses of 5,5'-dithiobis-(2-nitrobenzoic acid) (Ellman, 1959) or of copper sulphate abolished the E_{710} , rapidly in the former case and slowly in the latter. The possibility that iodoacetate reacted with the same thiol group as phenylmercuric acetate without disrupting the green complex can be eliminated, because in the presence of excess of iodoacetate small amounts of phenylmercuric acetate still 'de-greened' the enzyme.

Effect of potassium bromide. Attempts to reconstitute active enzyme by adding back FAD to the apoprotein have failed, although this has been achieved with the enzyme from ox liver (Mahler, 1954). FAD could be removed by dialysis at pH 6 against 0.1 M-potassium phosphate buffer containing 2 M-potassium bromide. The colourless apoprotein had an extinction peak at 280 nm. It did not bind FAD as judged by the absence of any quenching of fluorescence or reconstitution of catalytic activity.

Prolonged dialysis against water results in selective loss of FAD from the yellow form of the enzyme. It had been hoped that potassium bromide might accelerate the process and provide a means of obtaining the green form free of the yellow form. The reverse proved to be the case, however: an enzyme sample steadily 'de-greened' during dialysis against potassium bromide. Further investigation revealed that direct addition of potassium bromide to the enzyme without dialysis produced the same effect. As with other 'de-greening' procedures, decrease in E_{710} was associated with a rise in the main visible peak and a shift from 430 to 450 nm. Removal of the bromide by dialysis restored the E_{710}/E_{430} ratio to 60% of its original value, thus providing a second example of a reversible 'de-greening' procedure.

We have not explored the specificity of the bromide effect. However, very high concentrations of ammonium sulphate or of sodium or potassium phosphate are without effect, and it may be that only monovalent anions can 'de-green' in the same way as bromide. In agreement with Steyn-Parvé & Beinert's (1958) finding with the pig liver enzyme, we find no effect of 10 mM-potassium cyanide, but we have not examined the effect of very high concentrations of this salt.

DISCUSSION

Butyryl-CoA dehydrogenase from *P. elsdonii* has FAD as a prosthetic group, like the bovine liver enzyme. Its molecular weight appears to be about 150 000, with 3 or 4 flavin groups on each enzyme molecule. Amino acid analysis indicates a molecular weight of 43 400 per flavin group, and therefore perhaps the most likely structure is a tetramer

Fig. 6. Difference spectrum of green butyryl-CoA dehydrogenase upon treatment with excess of phenylmercuric acetate (6.3 mol/mol of enzyme-bound FAD) at room temperature. The difference spectrum was calculated from the total absorption spectra measured on the 0–0.5 slidewire of a Cary spectrophotometer. The extinctions of the untreated enzyme solution were 0.456 at 430 nm and 0.198 at 710 nm. The experiment was done at pH 7 in 0.1 M-potassium phosphate buffer.

of molecular weight 174000. This is probably within the error of the estimation of molecular weight by gel filtration, and is within the range estimated by Green, Mii, Mahler & Bock (1954) for the bovine enzyme from its sedimentation coefficient. The symmetry of the peak eluted from Sephadex columns suggests that any internal heterogeneity involves no great variation in molecular weight, in agreement with the results of Steyn-Parvé & Beinert (1958). However, the variable visible spectrum of the purified enzyme, taken together with the results of chemical reduction, clearly indicate the presence of at least two forms of the oxidized enzyme, a green and a yellow form. It will not be clear whether the green colour depends on flavin-flavin interaction, or whether a single enzyme molecule may contain both 'green' and 'yellow' subunits simultaneously, until physical means of resolving the green and yellow forms are found and active holoenzyme can be reconstituted from the apoprotein.

The results of chemical reduction suggest that in the green form of the enzyme the reactivity of the flavin is profoundly modified. The green form takes up more reducing equivalents per flavin group than the yellow form, but does so much more slowly. The catalytic activity shown in measurements of the initial rate of oxidation of butyryl-CoA is not, however, significantly altered, although a lag is detectable in the assay of the green enzyme without phenazine methosulphate. This merely indicates that the rate-limiting step in the assay is unaffected or else that the green complex dissociates under the assay conditions. This is discussed in the accompanying paper (Engel & Massey, 1971).

The spectral changes produced by sodium borohydride are strikingly similar to those observed with the amino acid oxidases by Massey *et al.* (1968) and with free flavins by Müller *et al.* (1969). The latter authors showed that the spectral shift is caused by formation of 3,4-dihydroflavin instead of the usual 1,5-dihydroflavin (the leuco form). Reduction by borohydride is accompanied in the case of the amino acid oxidases by covalent fixation of a molecule of substrate to the ϵ -amino group of a lysine residue (Coffey, Neims & Hellerman, 1965; Hellerman & Coffey, 1967; Massey *et al.* 1968). The enzyme is not inactivated by this procedure, however. Borohydride-treated D-amino acid oxidase displays a blue fluorescence, also observed with butyryl-CoA dehydrogenase. Whether substrate fixation occurs in the latter case is uncertain. This type of flavin reduction has not been found previously with flavoproteins other than the amino acid oxidases.

The spectrum obtained after borohydride reduction of the green form of butyryl-CoA dehydrogenase was clearly more complex than that obtained

with the yellow form, but can be simply accounted for by assuming that, although part of the green component was reduced, most of it remained intact while the yellow component was reduced. With the yellow enzyme, both the smallness of the 392 nm peak and the rapid appearance of an extinction at 450 nm on aeration suggest that reduction yielded a mixture of the usual 1,5-dihydroflavin and 3,4-dihydroflavin. The gradual return to a spectrum resembling that of the starting material is consistent with the observation (Müller *et al.* 1969) that 3,4-dihydroflavins are photo-oxidizable.

The wavelength of the extinction of butyryl-CoA dehydrogenase reduced by sodium borohydride is even lower (by about 15 nm) than those observed in the cases quoted above. The significance of this further hypsochromic shift is not clear.

The role of thiol groups in maintaining the green complex remains uncertain. Phenylmercuric acetate abolishes the peak at 710 nm without abolishing catalytic activity. Since arsenite is without effect, involvement of a dithiol can be excluded. There seem to be three possibilities: (a) direct interaction of a thiol group with the FAD; (b) direct interaction of a thiol group with another ligand that interacts with the FAD; (c) no direct involvement of thiol, but rather, in the presence of a bulky substituent, a steric or conformational effect preventing the juxtaposition of complexing partners. The catalytic activity of mercurial-treated enzyme is not readily compatible with the first possibility, but present evidence does not allow a decision between the others.

In this context the effect of copper sulphate is of some historical interest. The green colour of butyryl-CoA dehydrogenase was originally thought to be due to complexed copper (Mahler, 1954). Steyn-Parvé & Beinert (1958) showed, however, that copper could be removed with EDTA without altering the spectrum or the catalytic activity. In addition to their conclusive evidence, the present results show, for the bacterial enzyme at least, that Cu^{2+} ions actually abolish the green colour gradually.

In this preliminary characterization of butyryl-CoA dehydrogenase from *P. elsdenii* clear and important differences from the mammalian enzymes have been shown, especially with respect to redox behaviour. The similarities are nevertheless striking, and there can be little doubt that further work on this enzyme will yield valuable information applicable also to the mammalian systems.

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