A Study of the Subunit Structure and the Thiol Reactivity of Bovine Liver Uridine Diphosphate Glucose Dehydrogenase

By P. A. GAINEY,* T. C. PESTELL† and C. F. PHELPS*
*Department of Biochemistry, The Medical School, and †Molecular Enzymology Laboratory,
University of Bristol, Bristol BS8 1TD, U.K.*

1. The amino acid analysis of UDP-glucose dehydrogenase is reported. 2. N-Terminal-group analysis indicates only one type of N-terminal amino acid, methionine, to be present. 3. Peptide 'mapping' in conjunction with the amino acid analysis indicates that the subunits of the enzyme are similar if not identical. 4. The various kinetic classes of thiol group were investigated by reaction with 5,5'-dithiobis-(2-nitrobenzoate). 5. NAD$^+$, UDP-glucose and UDP-xylose protect the two rapidly reacting thiol groups of the hexameric enzyme. 6. Inactivation of the enzyme with 5,5'-dithiobis-(2-nitrobenzoate) indicates the involvement of six thiol groups in the maintenance of enzymic activity. 7. The pH-dependence of UDP-xylose inhibition of the enzyme was investigated. 8. The group involved in the binding of UDP-xylose to the protein has a heat of ionization of about 33kJ/mol and a pK of 8.4–8.6. 9. It is suggested that UDP-xylose has a cooperative homotropic effect on the enzyme.

UDP-glucose dehydrogenase (EC 1.1.1.22) catalyses the reaction:

$$\text{UDP-glucose} + 2\text{NAD}^+ \rightarrow$$
$$\text{UDP-glucuronic acid} + 2\text{NADH} + 2\text{H}^+$$

This reaction is unusual in that two moles of NAD$^+$ are utilized in the overall four-electron oxidation, whereas most dehydrogenases involving two-electron oxidation require only 1mol of NAD$^+$/mol of substrate. UDP-glucose dehydrogenase is similar in this respect to L-histidinol dehydrogenase (EC 1.1.1.23). Nelsestuen & Kirkwood (1971) have shown that the reaction may proceed as two individual two-electron oxidation steps involving an aldehyde intermediate.

Wilson (1965) has reported that the molecular weight of the bovine liver enzyme is about 300000. Zalitis & Feingold (1969) have shown that the enzyme consists of subunits of mol.wt. 52000. The enzyme is potently inhibited by thiol-group-modifying reagents (Strominger & Mapson, 1957) suggesting the involvement of a thiol group(s) in the maintenance of enzymic activity. Neufeld & Hall (1965) have shown the enzyme from various sources to be specifically and potently inhibited by UDP-xylose.

In the present paper we investigate in detail the subunit structure, and the role played by the thiol groups in the maintenance of enzyme activity.

Materials and Methods

Materials

Chemicals were obtained from the following: 2-mercaptoethanol and UDP-xylose from Sigma (London) Chemical Co., London S.W.6, U.K. NAD$^+$, NADH and UDP-glucose from Boehringer Corp. (London) Ltd., London W.5, U.K. TPCK-trypsin (3×crystallized, pretreated with 1-chloro-2-phenyl-3-toluene-*p*-sulphonamidobutan-2-one) from Worthington Biochemical Corp., Freehold, N.J., U.S.A.; Sephadex G-25 from Pharmacia, Uppsala, Sweden; Dns chloride, urea, *p*-dimethylamino-benzaldehyde, 8-hydroxyquinoline and ninhydrin from British Drug Houses Ltd., Poole, Dorset, U.K. The 5,5'-dithiobis-(2-nitrobenzoic acid) was from Calbiochem (London) Ltd., London W.1, U.K. Durable Polyamide thin-layer chromatograms were from Cheng-Chin Trading Co., Taipei, Taiwan, Republic of China. Whatman 3MM and no. 3 chromatography paper were from H. Reeve Angel and Co. Ltd., London E.C.4, U.K.

Urea was freed from ammonia and cyanate by passage through a column of Amberlite monobed resin MB-3. Bovine liver UDP-glucose dehydrogenase was purified to a specific activity of 3.5 by a slight modification of the method described by Zalitis & Feingold (1969). The protein gave a single band on polyacrylamide-gel electrophoresis. Dialysis tubing was from Visking Union Carbide, Chicago, Ill., U.S.A., and was boiled for 3–6h with three changes of double-distilled water before use.

Methods

Spectroscopic and kinetic measurements. The stopped-flow apparatus used was similar to that described by Gutfreund (1965). The interchangeable mixer and observation cell consisted of eight mixing jets and a 1cm light-path. Monochromatic light was

from a quartz–tungsten–iodine lamp and a Bausch and Lomb grating monochromator. Transmission of light was recorded on a Tektronix storage oscilloscope, which amplified the output of an EMI 9592B photomultiplier. The oscilloscope traces recorded percentage transmission on the ordinate axis. Reactions were studied at room temperature (21 ± 2°C). Unless otherwise stated the enzymic activity was assayed by using a 1 ml-capacity 1 cm-path-length cuvette containing 100μmol of sodium glycine buffer, pH 8.7, 1 μmol of NAD$^+$ and variable amounts of enzyme in a final volume of 1 ml. The reaction was started by the addition of 1 μmol of UDP-glucose and the increase in absorbance due to NADH production at 340 nm was measured in a Unicam SP. 1800 double-beam spectrophotometer with the appropriate blank. The sample cuvettes were brought to the operational temperature by preincubation for $2\frac{1}{2}$ min before the addition of UDP-glucose. Initial rates were recorded on a Servoscribe 'slave' recorder.

The 5,5'-dithiobis-(2-nitrobenzoate) solution was standardized in an excess of 2-mercaptoethanol and the E_{412} monitored. In these circumstances the ϵ is assumed to be 27.2 litre·mmol^{-1}·cm^{-1}.

The 2-chloromercuri-4-nitrophenol was synthesized as described by McMurray & Trentham (1969), and standardized in 0.1 M-NaOH, assuming ϵ 410 nm of 17.4 litre·mmol^{-1}·cm^{-1}.

Amino acid analysis. Freeze-dried enzyme (100μg) was resuspended in 100μl of 98–100 % formic acid and the mixture oxidized with 200μl (2 vol.) of precooled performic acid mixture [prepared by the method of Hirs (1967)] at 4°C for 2 h. The reaction was terminated by the addition of 3 ml of water, followed by freeze-drying. The residue was resuspended in 0.5 ml of 6.7 M-HCl and hydrolysed under vacuum for 24 h at 105°C. The hydrolysate was dried under vacuum with P_2O_5 and KOH. The residue was resuspended in 100μl of water and 20μl portions were applied to a column of C-4 Chromobead ion-exchange resin. The amino acid profile was established by using a Technicon TSM amino acid analyser with a citrate buffer system of increasing pH, according to the manufacturers instructions. A standard mixture of amino acids and a buffer blank were also run. The amino acid analysis was performed in quadruplicate. The mol of amino acid/mol of protein ratio was calculated by assuming a molecular weight of 300000 (Wilson, 1965; P. A. Gainey & C. F. Phelps, unpublished work).

Analysis for N-terminal group. About 3.3 nmol of enzyme (1.0 mg) was treated with Dns-chloride by the method of Gray (1967). After dansylation and protein hydrolysis, suitably sized samples of the dried hydrolysate were chromatographed on polyamide chromatography sheets by using the first two solvent systems and the method described by Woods & Wang (1967). The dried chromatogram was then eluted in

the original direction with a third solvent system, acetic acid–methanol–ethyl acetate (1:1:20, by vol.). A standard mixture of dansylated amino acids was also run and the individual dansylated amino acids identified as fluorescent spots when viewed under u.v. light.

Tryptophan determination. The tryptophan content of the protein was measured by the method of Goodwin & Morton (1946).

Peptide 'mapping'. The enzyme (3.0 mg) was heat-denatured at 100°C for 2 min. The precipitate was collected by centrifugation in a bench centrifuge, and resuspended in 2 ml of 0.5 % $(NH_4)HCO_3$, pH 8.0. TPCK-trypsin (0.06 mg, 1:50, w/w) was added to the resuspended material and the mixture incubated for 12 h at 37°C in a stoppered tube. The digestion mixture was freeze-dried. It was resuspended in 50μl of water and evenly applied to the origin on Whatman no. 3 chromatography paper (46 cm × 57 cm), allowing 1 cm/mg of protein digest. The chromatogram was developed with butan-1-ol–acetic acid–water (360: 105:535, by vol.), for 21 h. Phenol Red was run as a visible indicator of the extent of chromatography. After development, the chromatogram was air-dried overnight. The chromatogram was then subjected to high-voltage electrophoresis in the 90° vector, in pyridine–acetic acid–water (1:10:89, by vol.), pH 3.5, for 90 min at 2 kV, by using a Gilson high-voltage electrophoretor. A standard mixture of amino acids was also run along the edge of the chromatogram as reference markers. The chromatogram was air-dried and stained by the multiple dipping method of Jepson & Smith (1953). The chromatogram was dipped in a 1 % ninhydrin-cadmium acetate, and the ninhydrin-positive spots allowed to develop at room temperature overnight. After marking the position of the ninhydrin-positive spots the chromatogram was dipped in Ehrlich's reagent (Smith & Taylor, 1953) and the positions of the purple, tryptophan-containing peptides, which developed on air-drying, were noted. Finally the chromatogram was stained for arginine-containing peptides by dipping into a 0.1 % 8-hydroxyquinoline solution in acetone (Jepson & Smith, 1953), followed, after air-drying, by dipping into 100 ml of 0.5 M-NaOH, containing 20μl of bromine. On air-drying, arginine-containing peptides appeared as orange–red spots on a yellow background. The positions of the spots were noted. The dipping procedure used throughout did not result in a significant displacement of the peptide spots on the chromatogram.

Reaction of UDP-glucose dehydrogenase with 5,5'-dithiobis-(2-nitrobenzoate). Various concentrations of 5,5'-dithiobis-(2-nitrobenzoate) were incubated for 3 min at 30°C with 0.05 M-tris–HCl buffer, pH 7.5, in a final volume of 1 ml, in a Unicam SP. 1800 double-beam spectrophotometer. The reaction was initiated by the addition of 0.3 mg of enzyme (1 μM) to the

sample cuvette and the increase in production of the 2-nitro-5-thiobenzoate anion followed at 412 nm on a Servoscribe RE 511.20 potentiometric recorder with an excursion of 1.0 E units. In all cases the appropriate blank was used. In other experiments the 5,5'-dithiobis-(2-nitrobenzoate) concentration was varied in the presence of NAD$^+$ (1 mM) and UDP-glucose (1 mM) respectively.

An attempt was made to resolve the fast-reacting thiol groups by reaction of the enzyme with a tenfold excess of 5,5'-dithiobis-(2-nitrobenzoate). However, this method succeeded in resolving only the slower-reacting thiol groups. The total number of thiol groups was estimated with 5,5'-dithiobis-(2-nitrobenzoate) in the presence of 8 M-urea.

Loss of enzymic activity on thiol-group modification. Two experimental procedures were used; in the first 5,5'-dithiobis-(2-nitrobenzoate) was titrated, by using an automatic titrator, into 0.3 mg (1 μM) of enzyme in 1 ml of 0.05 M-tris–HCl buffer, pH 7.5. After each titration step, a suitably sized sample of the enzyme was taken, and assayed for enzymic activity. The titration was followed at 412 nm in a Unicam SP. 1800 spectrophotometer and was continued until the remaining enzymic activity approached a constant value. In the second method 0.3 mg (1 μM) of enzyme was treated with 6 μM- and 10 μM-5,5'-dithiobis-(2-nitrobenzoate) respectively, in a final volume of 1 ml of 0.05 M-tris–HCl buffer, pH 7.5. Suitably sized samples were removed at various times, and the percentage of original activity remaining was rapidly estimated by utilizing another SP. 1800 spectrophotometer, containing a preincubated assay mixture. Again activity measurements were performed until residual activity approached a constant value. In both cases, a plot was made of percentage original activity versus the number of thiol groups modified.

Stopped-flow studies of the rapidly reacting thiol groups. The fast-reacting thiol groups were investigated by using stopped-flow techniques. The reaction of the fast thiol-groups with 5,5'-dithiobis-(2-nitrobenzoate) was studied at 412 nm. This reaction was also studied in the presence of UDP-glucose (1 mM), NAD$^+$ (1 mM), NADH (0.1 mM and 1 mM) and UDP-xylose (80 μM). The fast-reacting thiol groups were also examined by using 2-chloromercuri-4-nitrophenol, and the reaction was monitored at 410 nm.

Effect of pH on UDP-xylose inhibition. The inhibition of UDP-glucose dehydrogenase by UDP-xylose was studied as a function of pH at 36°C by using 0.1 M-tris–HCl or 0.1 M-sodium glycine buffers over the pH range 8.1–9.1.

The heat of ionization of the group involved in the binding of UDP-xylose to the enzyme was estimated by observing the UDP-xylose inhibition as a function of pH at 26° and 36°C. The inhibition studies were performed by using 20 μmol of sodium pyrophosphate and 100 μmol of NaCl in a final volume of

1 ml, at pH values between 8.1–9.1. Results were presented as a plot of percentage inhibition or logarithm percentage inhibition versus pH.

Results

Amino acid analysis

The amino acid composition of the protein, expressed as the nearest whole number of individual amino acids is given in Table 1. Glutamine, asparagine, glutamate and aspartate were not estimated. No correction factors have been applied for the destruction of serine and threonine, or for the slow release of valine and isoleucine.

An amino acid analysis in which the initial performic acid oxidation step was omitted showed no cysteic acid. The number of arginine and lysine residues present/50000 molecular weight subunit were 26 and 28 respectively, and the total number of arginine and lysine residues was 54. The number of cysteine residues, estimated as cysteic acid was 72.

Tryptophan determination

A total of 27 tryptophan residues/300000 g of enzyme were found. This corresponds to about five

Table 1. *Amino acid composition of bovine liver UDP-glucose dehydrogenase*

The amino acid composition was the average of four samples. Cysteine was determined as cysteic acid, and methionine as its sulphone, after performic acid oxidation of the protein. Tryptophan was estimated by the method of Goodwin & Morton (1946).

Amino acid	No. of amino acid residues/300000 g of protein
Cysteine	72
Methionine	44
Threonine	145
Serine	171
Proline	145
Glycine	193
Alanine	228
Valine	168
Isoleucine	173
Leucine	180
Tyrosine	47
Phenylalanine	90
Histidine	77
Arginine	156
Lysine	168
Glutamate and glutamine	243
Aspartate and asparagine	289
Tryptophan	27
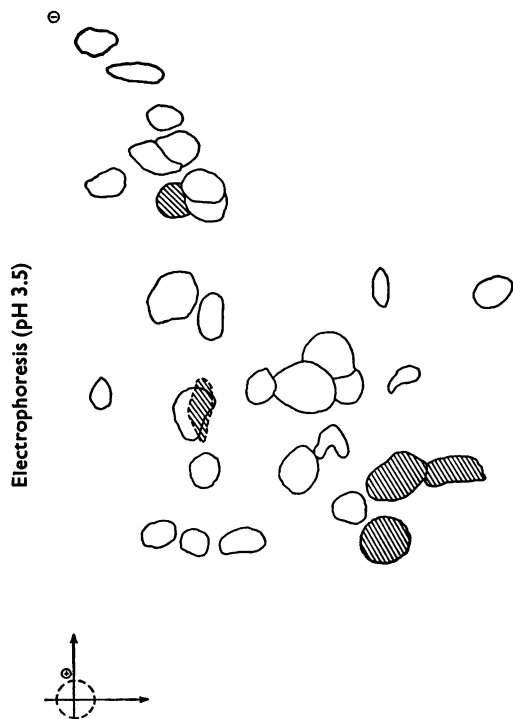
Fig. 1. *Peptide 'map' after development with acidic Ehrlich's reagent for tryptophan-containing peptides (hatched spots) followed by development with 8-hydroxyquinoline for arginine-containing peptides (unshaded spots)*

For details see the text.

Fig. 2. *Reaction of 5,5'-dithiobis-(2-nitrobenzoate) with bovine liver UDP-glucose dehydrogenase*

●, Enzyme (0.3 mg) with 0.5 mM-5,5'-dithiobis-(2-nitrobenzoate); o, enzyme (0.3 mg) plus UDP-glucose (1 mM) with 0.5 mM-5,5'-dithiobis-(2-nitrobenzoate); ■, enzyme (0.3 mg) plus NAD^+ (1 mM) with 0.5 mM-5,5'-dithiobis-(2-nitrobenzoate). The temperature was 31°C. The reactions were performed in a final volume of 1 ml of 0.1 M-tris–HCl buffer, pH 7.5. After 1 h no further increase in E_{412} was observed and the total E change was taken as the end-point of the reaction.

tryptophan residues/50000 molecular weight sub-unit, in agreement with the number five of trypto-phan-positive peptides found after peptide 'mapping' and treatment with acidic Ehrlich's reagent (Fig. 1).

Peptide 'map'

A typical peptide 'map' obtained on staining with the ninhydrin–cadmium acetate mixture is shown in Plate 1. About 60 peptide spots were revealed, in good agreement with the 55 peptide spots expected from the total arginine and lysine content of the pro-tein, as determined by amino acid analysis. The extra peptide spots may represent peptide 'overlaps' formed by incomplete tryptic hydrolysis of poorly susceptible bonds. On development with the modified Sakaguchi reagent a total of 26 orange–red spots (Fig. 1) were observed. This was in excellent agreement with the expected 26 spots predicted from the amino acid analysis. Little or no material remained at the origin, even after chlorination and subsequent
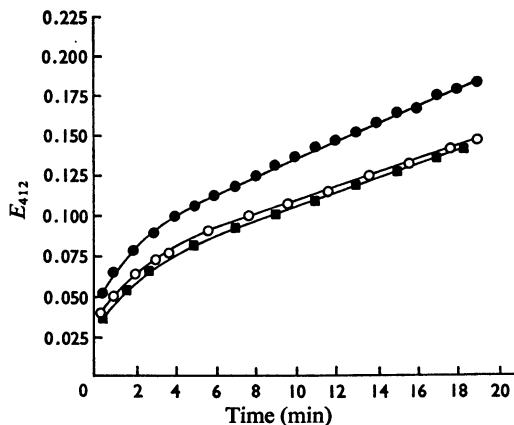
development of the peptide maps with o-toluidine–KI reagent. The Sakaguchi reagent also revealed little or no peptide material at the origin.

N-Terminal amino acid analysis

Apart from fluorescent spots corresponding to Dns-ε-lysine and Dns-O-tyrosine, the only other major Dns-amino acid observed was Dns-methio-nine. It is assumed therefore that methionine is the only N-terminal amino acid. A similar result was obtained by Uram (1971) who found 0.7 mol of methionine/50000 g of protein.

Reaction with 5,5'-dithiobis-(2-nitrobenzoate)

A typical reaction profile with the enzyme is shown in Fig. 2. An initial transient corresponding to about two rapidly reacting thiol groups was observed. Following the initial fast phase, a slower phase corresponding to about four thiol groups was ob-served. Finally, a slower, somewhat linear phase, corresponding to 26–30 thiol groups, was observed. In all, a total of 32–36 thiol groups reacted with the 5,5'-dithiobis-(2-nitrobenzoate). Addition of sodium dodecyl sulphate to a final concentration of 0.2% resulted in the reaction of a further 11–15 thiol groups.
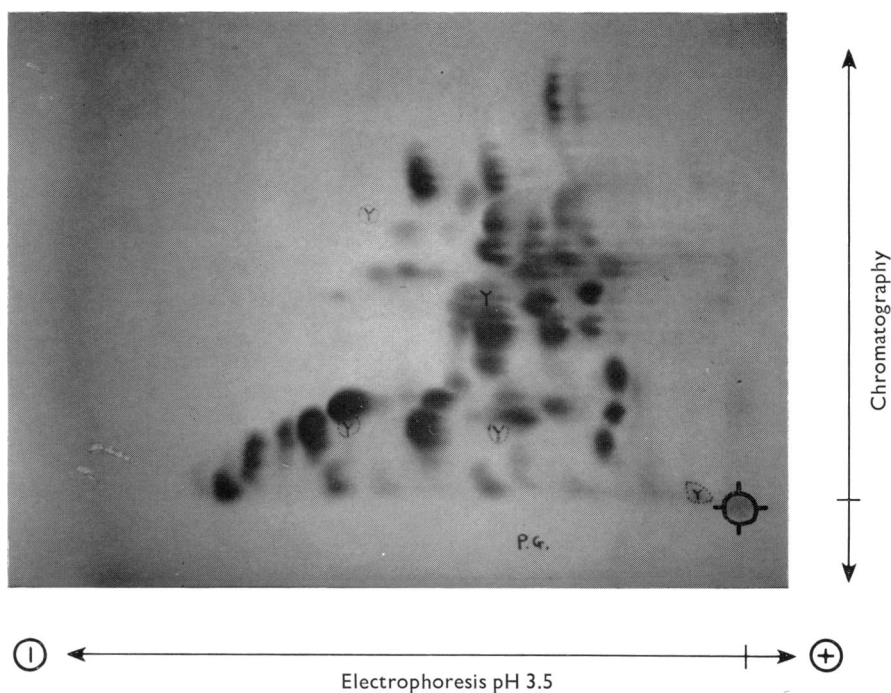
EXPLANATION OF PLATE I

Plate 1. *Peptides formed by trypsin digestion of heat denatured bovine liver UDP-glucose dehydrogenase*

The peptide 'map' was prepared by paper chromatography and electrophoresis and stained with ninhydrin–cadmium acetate mixture as described in the Materials and Methods section. Areas marked 'Y' indicate yellow-staining peptide spots. The Phenol Red and amino acid markers are not shown.

On increasing the sodium dodecyl sulphate concentration to 0.5% a further 18–19 thiol groups were exposed for reaction. With 6M-urea, the protein
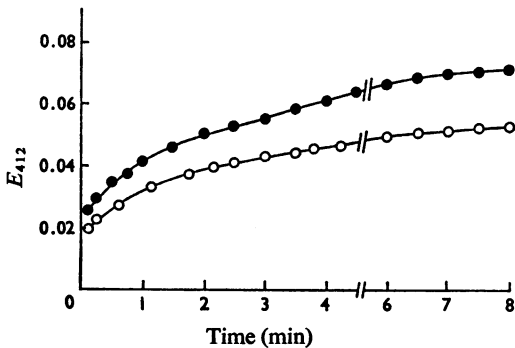

Fig. 3. Reaction of 5,5'-dithiobis-(2-nitrobenzoate) with bovine liver UDP-glucose dehydrogenase

●, Enzyme (0.3 mg) with 10 μM-5,5'-dithiobis-(2-nitrobenzoate); o, enzyme (0.3 mg) with 6 μM-5,5'-dithiobis-(2-nitrobenzoate). Reactions were performed in a final volume of 1 ml of 0.1 M-tris–HCl buffer, pH 7.5. The temperature was 29.5°C.

yielded a total of 72 thiol groups reacting with 5,5'-dithiobis-(2-nitrobenzoate). This number could not be further increased by the presence of 8 M-urea.

In the presence of NAD$^+$ (1 mM), a total of two thiol groups were protected from reaction with 5,5'-dithiobis-(2-nitrobenzoate), further, the two protected thiol groups appeared to correspond to the two rapidly reacting thiol groups (Fig. 2). UDP-glucose (1 mM) had a similar effect (Fig. 2) in appearing to protect the two rapidly reacting thiol groups.

The reaction profile shown in Fig. 3 shows the reaction of enzyme with lower concentrations of 5,5'-dithiobis-(2-nitrobenzoate). The initial fast-reacting thiol groups are seen much more clearly and again correspond to about two thiol groups.

Enzyme inactivation with 5,5'-dithiobis-(2-nitrobenzoate)

The effect of titrating the enzyme with increasing amounts of 5,5'-dithiobis-(2-nitrobenzoate) is shown in Fig. 4(a). Extrapolation from the initial slope yields an estimate of six thiol groups/molecule as being essential for enzymic activity. Fig. 4(b) shows the same type of plot obtained by removing samples of an enzyme–5,5'-dithiobis-(2-nitrobenzoate) reaction
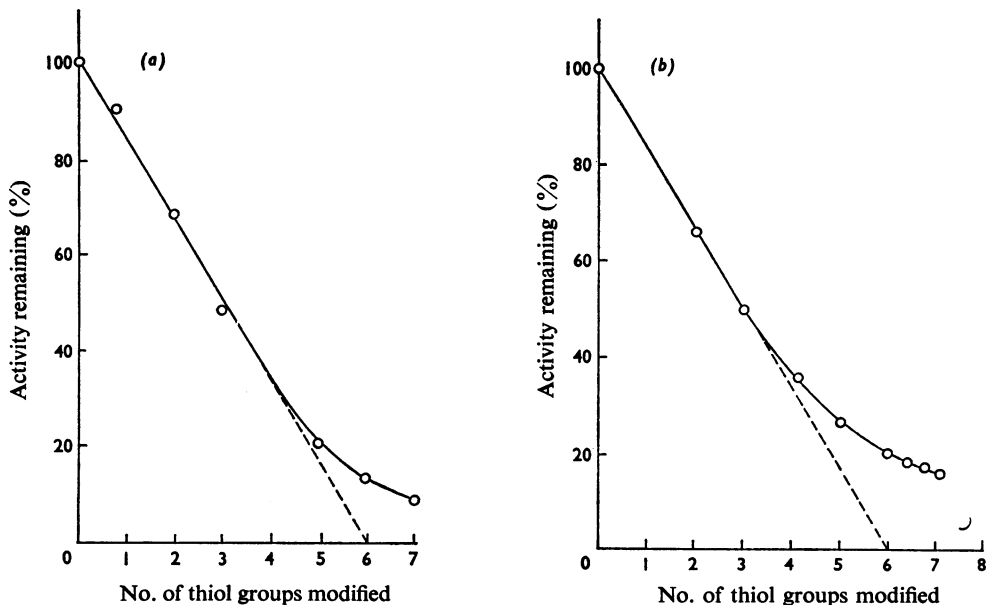

Fig. 4. Inactivation of bovine liver UDP-glucose dehydrogenase by reaction with 5,5'-dithiobis-(2-nitrobenzoate)

(a) Titration of the enzyme (0.3 mg) in a final volume of 1 ml of 0.1 M-tris–HCl buffer, pH 7.5, with 5,5'-dithiobis-(2-nitrobenzoate). Suitably sized samples were removed and assayed for residual enzymic activity. (b) Sampling of an enzyme (0.3 mg)–10 μM-5,5'-dhthiobis-(2-nitrobenzoate) reaction mixture in a final volume of 1 ml of 0.1 M-tris–HCl buffer, pH 7.5. Suitably sized samples were removed at various times and assayed for residual enzymic activity. The temperature was 30°C.

mixture at various times. Again, extrapolation from the initial slope, indicated that six thiol groups/molecule are required for enzymic activity.

Stopped-flow studies

The spectrophotometric record shown in Fig. 5(a) represents the reaction of 0.85 μM-enzyme with 10 μM-5,5'-dithiobis-(2-nitrobenzoate). The amplitude of the transient phase was determined as the change in percentage transmission between the observed trace and the linear portion of the initial trace both extrapolated back to zero time. This was about 4.4% transmission at 412 nm and corresponds to about 1.8 thiol groups. The actual value was assumed to be two. The rate constant of the initial transient was evaluated by using Sturtevant's modification of Guggenheim's method (Sturtevant, 1937) and was 0.44s⁻¹. With NAD⁺ (1 mM) the initial transient was not observed (Fig. 5b); instead, the trace resembled a 'slow drift' which could not be resolved to give a simple exponential function. It was not possible to superimpose this trace on the latter part of the trace in Fig. 5(a), that is that part which follows the initial transient. Fig. 6(a) shows the reaction in the presence of 1 mM-UDP-glucose. As with NAD⁺, the initial transient was not observed; also, the slow drift was not resolvable and could not be superimposed on that shown in Fig. 5(a). The presence of 80 μM-UDP-xylose has the effect shown in Fig. 6(b). Here again the initial transient was not observed; however, the trace could be superimposed on the trace obtained in the presence of UDP-glucose (Fig. 6a). NADH (100 μM) had the effect shown in Fig. 5(c). The initial transient was still observable. On raising the NADH concentration to 1 mM, however, the initial transient disappeared.

The spectrophotometric record of the reaction of 0.425 μM-protein with 10 μM-2-chloromercuri-4-nitrophenol is shown in Fig. 7. The ΔE_{410} was 8.8%. The horizontal trace indicates the percentage transmission recorded up to 30s after mixing, when no further increase was apparent. Because of the non-homogeneous character of the trace it was not possible to resolve a simple exponential function, or to calculate the first-order rate-constant. The total amplitude was utilized to calculate the number of thiol groups reacting, assuming that the ionization of the phenolate ion, when bound to the protein, approximates to that when bound to thioglycollic acid, i.e. at pH7.0, ϵ is 5.57 litre·mmol⁻¹·cm⁻¹. A total of 22 thiol groups appear to be reacting. After the reaction of the thiol groups with the mercurial was complete, a slight decrease in percentage transmission was observed, which appeared to be associated with protein denaturation and precipitation. Experiments similar to that described above were repeated in the presence of each of the following:
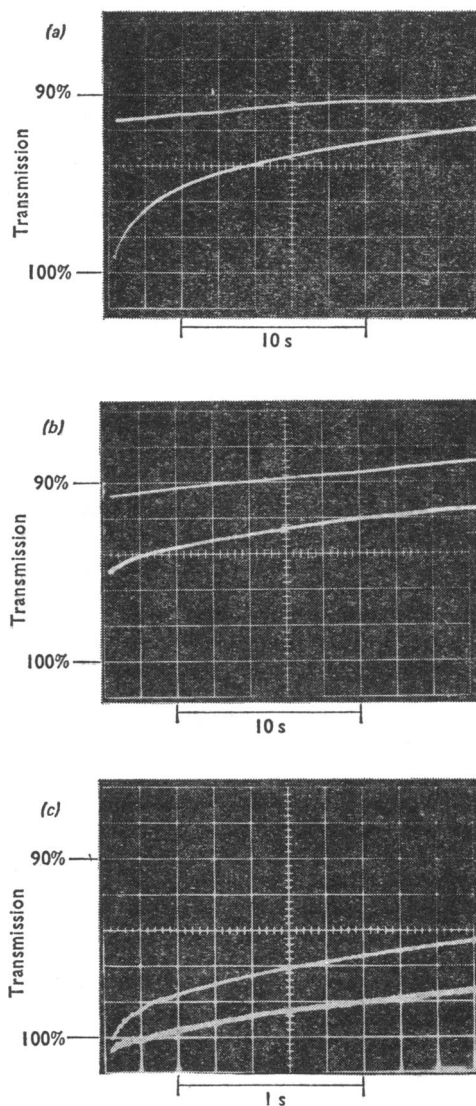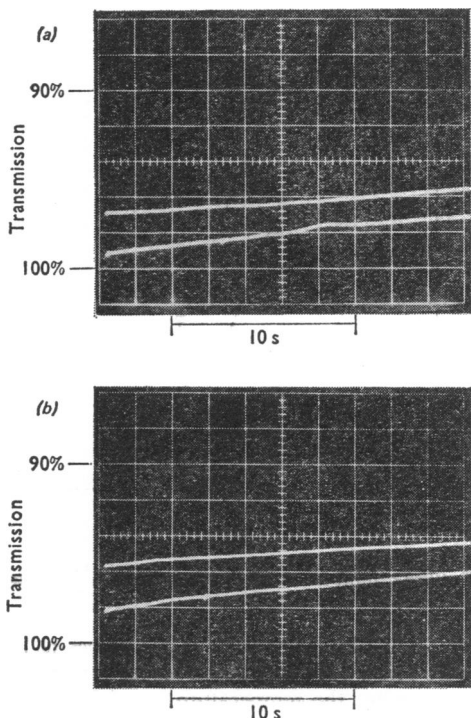

Fig. 5. *Stopped-flow spectrophotometric record at 412 nm of the reaction between UDP-glucose dehydrogenase and 5,5'-dithiobis-(2-nitrobenzoic acid) at 21°C in tris(hydroxymethyl)aminomethane (100 mM) adjusted to pH8.0 with HCl*

The upper trace in Fig. 5(a) and 5(b) is a record of the reaction after the completion of the initial trace. (a) Reaction of UDP-glucose dehydrogenase(0.85 μM) and 5,5'-dithiobis-(2-nitrobenzoic acid) (10 μM); (b) reaction (a) repeated in the presence of NAD⁺ (1.0 mM); (c) reaction of UDP-glucose dehydrogenase (0.5 μM) and 5,5'-dithiobis-(2-nitrobenzoic acid) (50 μM) in the presence of NADH (1.0 and 0.1 mM). The lower trace corresponds to the reaction in the presence of NADH (1.0 mM).

Fig. 7. *Stopped-flow spectrophotometric record at 410nm of the reaction between UDP-glucose dehydrogenase (0.425 μM) and 2-chloromercuri-4-nitrophenol (10.0 μM) at 21°C in tris(hydroxymethyl)aminomethane (100mM) adjusted to pH7.0 with HCl*

The horizontal lower trace was obtained a few seconds after the end of the reaction.

Fig. 6. *Stopped-flow spectrophotometric record at 412nm of reaction between UDP-glucose dehydrogenase and 5,5'-dithiobis-(2-nitrobenzoic acid) at 21°C in tris(hydroxymethyl)aminomethane (100mM) adjusted to pH8.0 with HCl*

The upper trace is a record of the reaction after the completion of the initial trace. (a) Reaction of UDP-glucose dehydrogenase (0.85 μM) and 5,5'-dithiobis-(2-nitrobenzoic acid) (10 μM) in the presence of UDP-glucose (1.0mM); (b) reaction (a) repeated in the presence of UDP-xylose (0.08mM).

1 mM-NAD$^+$, 1 mM-UDP-glucose, 80 μM-UDP-xylose. In each case, the trace could be superimposed on that shown in Fig. 7 although some difference in the total amplitude was observed. Again a slight positive drift after the completion of the reaction indicated that the protein was precipitating.

Effect of pH on UDP-xylose inhibition

Fig. 8 shows a plot of percentage inhibition versus pH in tris–HCl buffer. A sigmoid curve is obtained, with a pK at the inflexion point of 8.4–8.6. Fig. 8 also shows that, whereas the percentage inhibition increased rapidly with decrease in pH, the enzymic activity decreased very little over the same pH
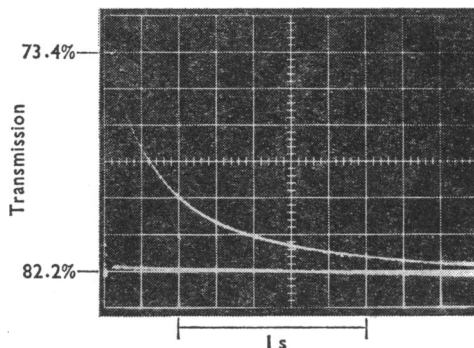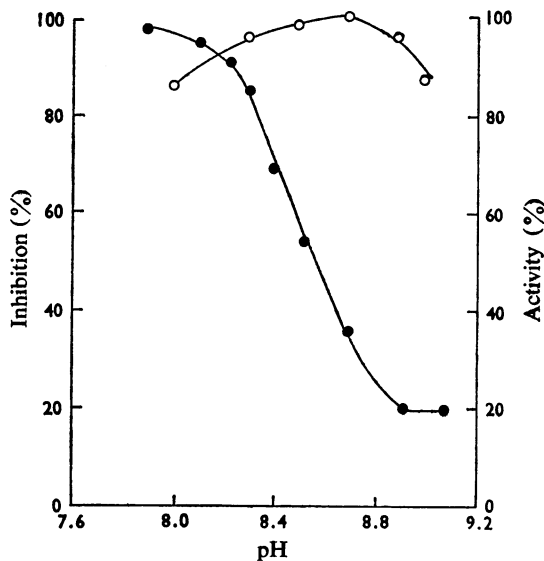

Fig. 8. *Effect of pH on UDP-xylose inhibition of bovine liver UDP-glucose dehydrogenase*

●, Percentage inhibition in 0.1M-tris–HCl buffer at various pH values. The UDP-glucose, NAD$^+$ and UDP-xylose concentrations were 1mM, 1mM and 20 μM respectively; o, percentage activity in 0.1M-tris–HCl buffer at various pH values. The concentrations of UDP-glucose and NAD$^+$ were both 1mM. The temperature was 36°C.
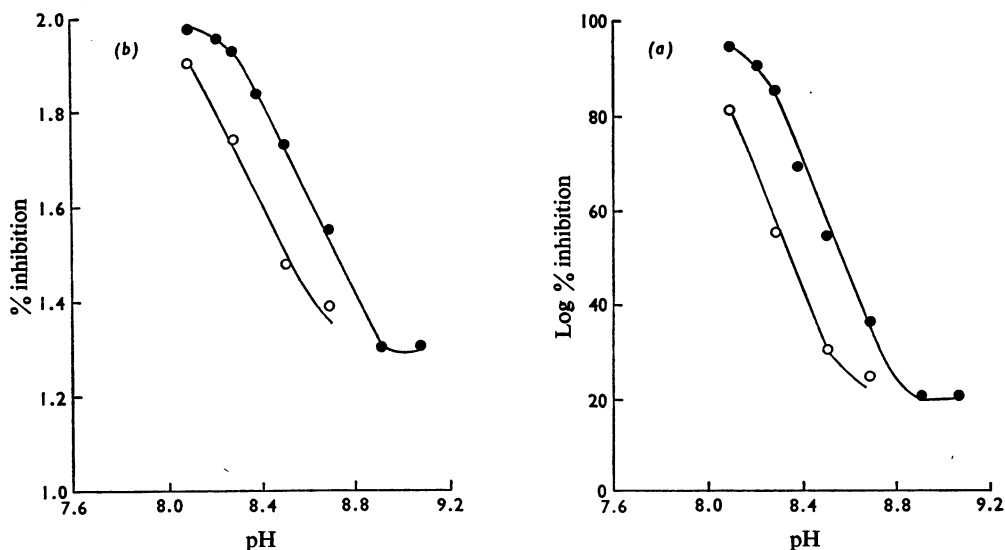
Fig. 9. *Effect of temperature on the pH-dependence of UDP-xylose inhibition of bovine liver UDP-glucose dehydrogenase*

●, Percentage inhibition in 20mM-sodium pyrophosphate–100mM-NaCl buffer at various pH values at 36°C. The concentrations of NAD⁺, UDP-glucose and UDP-xylose were 1mM, 1mM and 20μM respectively; o, percentage inhibition obtained under the same conditions as above, except the temperature was 26°C. (*b*) is a logarithmic plot of (*a*).

range. Much larger concentrations of UDP-glucose were required to remove the UDP-xylose inhibition at lower pH, than at higher pH. The percentage inhibition was lowered by raising the pH with solid tris.

Heat of ionization of the group involved in UDP-xylose binding to the protein

The effect of temperature on UDP-xylose inhibition as a function of pH is shown in Fig. 9(*a*). The inhibition studies were performed at two different temperatures by using 20mM-sodium pyrophosphate–100mM-NaCl buffer. Sodium pyrophosphate was used because of its negligible heat of ionization. At both temperatures, a plot of percentage, or log percentage inhibition versus pH, yielded sigmoid profiles. From Fig. 9(*b*) the apparent heat of ionization Q' may be calculated utilizing the relationship $Q' = -2.303RT^2 \, d(\mathrm{pH})/dT$ (Wyman, 1939) where d(pH) is the change in pH, and dT the mean temperature (31°C). A plot of Q' versus the average pH yields a plateau, indicating a Q' value of about 33.5kJ/mol.

Discussion

The number of ninhydrin-positive peptide spots and also the number of arginine- and tryptophan-

containing peptide spots observed after development with ninhydrin–cadmium acetate, 8-hydroxyquinoline and Ehrlich reagents respectively, are in good agreement with the number of peptides predicted from the amino acid analysis, assuming the enzyme to consist of identical subunits. Bearing in mind the pitfalls inherent in the interpretation of peptide 'maps' (Harris & Hindley, 1965; Fraenkel-Conrat, 1965), it seems most probable that the UDP-glucose dehydrogenase approximates to a homopolymer or a close variant thereof. Evidence for this is that only one *N*-terminal amino acid, methionine, appears to be present, and that only one band is seen on polyacrylamide-gel electrophoresis in the presence of sodium dodecyl sulphate (Zalitis & Feingold, 1969; P. A. Gainey & C. F. Phelps, unpublished work) and in sedimentation-equilibrium studies in 6M-guanidine hydrochloride (Uram, 1971).

The small amount of peptide-containing material remaining at the origin after electrophoresis indicates the absence of an insoluble core, and is a noteworthy feature of UDP-glucose dehydrogenase. The peptide 'maps' may therefore be interpreted with the knowledge that the visible peptides observed represent most, if not all, of the amino acids of the parent protein. In this respect, UDP-glucose dehydrogenase contrasts markedly with other proteins such as chymotrypsin (Hartley, 1964), and turnip yellow

mosaic virus (Harris & Hindley, 1965), where as much as 70% of these proteins formed insoluble cores, which were not resolvable by paper chromatography and electrophoresis.

The total number of thiol groups (72) estimated by amino acid analysis, and by reaction with 5,5′-dithiobis-(2-nitrobenzoate) in the presence of 8M-urea are in good agreement, and indicate that there are no disulphide bridges in this enzyme. This number corresponds to 12 thiol groups per subunit, assuming a subunit molecular weight of about 50000 (P. A. Gainey & C. F. Phelps, unpublished work; Zalitis & Feingold, 1969). The progressive unfolding of the enzyme, as judged by the increased number of thiol groups exposed for reaction with 5,5′-dithiobis-(2-nitrobenzoate) on increasing the concentration of dissociating agents (sodium dodecyl sulphate and urea) is also consistent with the absence of disulphide bonds.

Reaction of the enzyme with 5,5′-dithiobis-(2-nitrobenzoate) showed the presence of several kinetic classes of thiol groups. NAD^+, and UDP-glucose and UDP-xylose caused the disappearance of the initial transient (corresponding to two thiol groups) observed with stopped-flow techniques. It may be concluded that these thiol groups are involved primarily in the reactivity of the enzyme, associated either with the binding of substrates or with the conformational stability of the enzyme. Modification with 5,5′-dithiobis-(2-nitrobenzoate) also yielded a value of six thiol groups/hexamer as being essential for the maintenance of enzyme activity (Fig. 4a). Following the two fast-reacting thiol groups four somewhat slower-reacting thiol groups were observed (Fig. 2). Whereas the two fast-reacting thiol groups appear to be involved at or near the site of NAD^+ or UDP-glucose binding the function of the four medium-fast-reacting thiol groups is as yet not clear. Two possibilities exist. First, the four thiol groups may represent another two potential active sites which come into effect after the initial enzyme turnover, or secondly the four thiol groups may function in the overall maintenance of the conformation of the active site. It is assumed that the two fast-reacting and the four medium-fast-reacting thiol groups together constitute the six thiol groups that are essential for the maintenance of enzymic activity.

Unexpectedly UDP-glucose also protects the fast-reacting thiol groups. A comprehensive steady-state analysis indicated a mechanism with compulsory order of addition (P. A. Gainey & C. F. Phelps, unpublished work), the NAD^+ binding before UDP-glucose. The mechanism by which UDP-glucose protects the fast-reacting thiol groups is unknown at present, though it is possible that UDP-glucose has a finite affinity for the enzyme even when NAD^+ is absent, which is sufficient to afford some protection of these fast-reacting thiol groups. The observation

that UDP-glucose and NAD^+ both protect the same fast-reacting thiol groups may indicate that both of these molecules are bound in close proximity at the active site. By using stopped-flow techniques it was found that the slower phases of thiol reaction, after the initial transient in the presence of NAD^+ and UDP-glucose respectively (Figs. 5b and 6a) were not superimposable. This may be explained by assuming that the binding of either NAD^+ or UDP-glucose to the enzyme each results in different conformational effects leading to a difference in reactivity of the 'slower phase' thiol groups. That the slower phases observed in the presence of UDP-xylose or UDP-glucose are superimposable is to be expected because of the very similar structures of these compounds. UDP-xylose would be expected to be a competitive inhibitor with respect to UDP-glucose and hence to protect the two fast-reacting thiol groups. In the presence of $100\,\mu$M-NADH the two rapid-reacting thiol groups are observed (Fig. 5c) and it may be concluded that NADH does not protect these thiols under the conditions employed. On raising the NADH concentration to 1mM, the initial transient was not observed.

After the reaction of the initial six thiol groups, 26–30 slower-reacting thiol groups were observed. The rate of reaction of this group of thiols with both high and low concentrations of 5,5′-dithiobis-(2-nitrobenzoate) was very similar, indicating that the unfolding of the protein is the rate-limiting step. The exact function of these thiol groups is unknown. Dissociation of the protein subunits with increasing concentrations of sodium dodecyl sulphate and urea, showed 36–40 thiol groups to be buried within the protein.

An investigation of the reactivity of the fast-reacting thiol groups by using 2-chloromercuri-4-nitro-phenol was unsuccessful because the reagent did not differentiate between the thiol groups, and this resulted in a non-homogeneous reaction profile (Fig. 7) which could not be resolved into simpler exponential functions. The difference in reactivity observed between the mercurial and 5,5′-dithiobis-(2-nitrobenzoate), may be attributed to the fact that the disulphide bond between the cysteine residue of the protein and the 2-nitro-5-thiobenzoate group involves a dihedral angle of 90° between the carbon-sulphur bonds (Calvin, 1954). This imposes a steric restriction on the accessability of the thiol group to reaction with 5,5′dithiobis-(2-nitrobenzoate). In marked contrast to this, the linear mercury-sulphur-carbon bond formed by the organo-mercurial adduct exerts no discrimination in the thiol groups attacked (Gutfreund & McMurray, 1971).

The effect of pH on UDP-xylose inhibition is shown in Fig. 8. Whereas the uninhibited rate remains approximately constant over the pH range 8.1–9.1, the UDP-xylose inhibition increases rapidly on

decreasing the pH. The pK value determined from the inflexion point of the curve (Fig. 8) is 8.4–8.6 at 26°C. A similar effect has been observed with sheep nasal-septum cartilage UDP-glucose dehydrogenase (Gainey & Phelps, 1972). The apparent heat of ionization, Q', of the group involved in the binding of UDP-xylose to the protein was calculated to be about 33 kJ/mol. Borsook et al. (1937) have reported a pK_2 value of 8.33 for cysteine, whilst Benesch & Benesch (1955) have shown the heat of ionization for the thiol group of thioglycollic acid to be 28.9 kJ/mol. According to Tanford (1962) the pK of the imidazole moiety of several proteins is in the range 6.4–7.4. Similarly the heat of ionization of the imidazole group in proteins may be between 29.3 and 33.5 kJ/mol. The heat of ionization of the imidazole group has been shown by Nozaki et al. (1957) to be 32.2 kJ/mol.

Overall, the above data would suggest the involvement of a thiol or an imidazole group in the binding of UDP-xylose to the protein. One should be warned, however, against matching the value of Q' and pK for one type of group in a protein molecule, with the same type of group in a simple compound. The observations that UDP-xylose protects the initial transient thiol groups (Fig. 6b), and also that the slower phases of thiol reaction following the initial transient in the presence of UDP-glucose or UDP-xylose are superimposable, would argue for the binding of UDP-xylose at the UDP-glucose site. The similar structures of the two compounds would indicate a competitive mode of inhibition by UDP-xylose with respect to UDP-glucose. Such a mode of inhibition has been observed (Neufeld & Hall, 1965; Bdolah & Feingold, 1968; P. A. Gainey & C. F. Phelps, unpublished work). The absence of a bulky C-6 primary alcohol group might allow UDP-xylose to bind at the UDP-glucose site with equivalent, if not greater affinity than UDP-glucose. On adding UDP-xylose to UDP-glucose dehydrogenase in fluorescence-titration experiments no protein fluorescence perturbation was observed. However, when NAD$^+$ and enzyme were similarly titrated with UDP-xylose a substantial fluorescence perturbation was seen (P. A. Gainey & C. F. Phelps, unpublished work). Since a steady-state analysis revealed an ordered addition of substrates to the enzyme (NAD$^+$ binding before UDP-glucose), and also as UDP-xylose is a close structural analogue of UDP-glucose, as described above, it is probable that UDP-xylose exerts its inhibitory effect at the UDP-glucose-binding site. If confirmed, this would suggest that UDP-xylose is a true competitive inhibitor with respect to UDP-glucose and in so being exhibits a co-operative homotropic effect and not an allosteric effect on the enzyme, as postulated by

Neufeld & Hall (1965). Such a mechanism of inhibition would explain the observed Hill's coefficient of 2.3 (Neufeld & Hall, 1965; P. A. Gainey & C. F. Phelps, unpublished work) indicating substantial co-operativity between the subunits of the enzyme.

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