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Use of Dimethyl Suberimidate and Novel Periodate-Cleavable Bis(imido esters) to Study the Quaternary Structure of the Pyruvate Dehydrogenase Multienzyme Complex of *Escherichia coli*†

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ABSTRACT: Two new symmetrical bis(imido esters), *N,N'*-bis(2-carboximidoethyl)tartarimide dimethyl ester dihydrochloride and *N,N'*-bis(2-carboximidomethyl)tartarimide dimethyl ester dihydrochloride, have been synthesized. Tests with the tetrameric enzyme, fructose diphosphate aldolase, show that these reagents closely resemble dimethyl suberimidate in their ability to cross-link protein subunits. However, identification of the cross-linked species, separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis, is greatly facilitated since the cross-links can be broken by a simple treatment with sodium periodate. The periodate cleavage step can be introduced between the two dimensions of a diagonal gel electrophoretic separation, the contributors to a cross-linked species then moving off the diagonal formed by uncross-linked proteins and reverting to the positions in the gel that correspond with their regenerated monomeric form. When the pyruvate dehydrogenase multienzyme complex of *Escherichia coli* was treated with dimethyl suberimidate or *N,N'*-bis(2-carboximidoethyl)tartarimide dimethyl ester dihydrochloride, cross-links rapidly formed between the subunits of the transacetylase and lipoamide dehydrogenase components. On the other hand, cross-links failed to form between the subunits of the decarboxylase component themselves, or between the decarboxylase and the other two types of subunit in the complex. Cross-linking experiments with the isolated lipoamide dehydrogenase were compatible with the accepted dimeric structure of this enzyme in free solution, whereas the isolated pyruvate decarboxylase component also failed to cross-link when treated with dimethyl suberimidate in free solution. The cross-linking experiments with the intact multienzyme complex provide evidence for the existence of the lipoamide dehydrogenase dimer in the assembled enzyme and show the need to interpret such experiments with care since, from other evidence, the pyruvate decarboxylase component is known to be bound to the transacetylase "core" of the complex.

The pyruvate dehydrogenase multienzyme complex of *Escherichia coli* contains three different types of polypeptide chain, responsible for the three component enzyme activities; these are: E1, pyruvate decarboxylase; E2, lipoyl transacetylase; and E3, lipoamide dehydrogenase. The component enzymes can be taken apart and reassembled in vitro to form the original structure which is somewhat larger than a ribosome (for reviews, see Reed and Oliver (1968) and Reed (1974)). The intact enzyme and the lipoyl transacetylase "core" probably have octahedral symmetry (Reed, 1974), but the subunit structure is still a matter of some controversy (Eley et al., 1972; Vogel et al., 1972; Perham, 1975; Bates et al., 1975). The number of polypeptide chains in the complex has been reported to be 48 (Vogel et al., 1972) or 60 (Eley et al., 1972).

Cross-linking reactions have proved helpful in analyzing the geometrical arrangements of protein subunits in a wide variety of situations, such as oligomeric enzymes (Cohlberg et al., 1972), membranes (Ji and Ji, 1974), ribosomes (Bickle et al., 1972), and histones (Kornberg and Thomas, 1974). Reagents based on bis(imido esters), e.g. dimethyl suberimidate (Davies and Stark, 1970), have proved particularly useful since they react specifically with protein amino groups and yield stable products. The reaction causes no change of charge at the modified residue, and the tertiary and quaternary structures of the proteins are therefore likely to remain unaffected (Perham, 1973).

The principal method of analyzing the products of cross-linking reactions has been sodium dodecyl sulfate gel electrophoresis in which the various species are characterized by measuring their molecular weights. However, in studies of complex systems, ambiguities in identifying the protein chains contributing to a cross-linked species must inevitably arise. To help overcome this difficulty, the use of bis(imido esters) containing a disulfide bond has been described (Sun et al., 1974; Wang and Richards, 1974). The disulfide bond can readily be cleaved after an initial electrophoretic separation of cross-linked species; this breaks the cross-links and permits the regenerated monomers to be characterized in a second dimension of gel electrophoresis. The strategy is therefore akin to that of diagonal paper electrophoresis of peptides (Hartley, 1970; Perham, 1969).

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[1] Abbreviations used are: CETD, *N,N'*-bis(2-carboximidoethyl)tartarimide dimethyl ester dihydrochloride; CMTD, *N,N'*-bis(2-carboximidomethyl)tartarimide dimethyl ester dihydrochloride; EDTA, (ethylenedinitrilo)tetraacetic acid.

There are several potential difficulties with the use of bis(imido esters) that contain disulfide bridges. First, they cannot be employed under reducing conditions, which precludes their use with many enzyme systems that are sensitive to oxidation. Secondly, there is the possibility of disulfide interchange occurring during the handling of the cross-linked protein before gel electrophoresis. This could confuse the pattern of cross-linking established in the intact structure and so invalidate the use of these reagents as probes of native structure. Thirdly, the desirable treatment of protein samples with 2-mercaptoethanol before analysis by sodium dodecyl sulfate gel electrophoresis (Shapiro and Maizel, 1969) is necessarily excluded. To avoid these difficulties, we have sought a more generally applicable reagent. This has led us to synthesize *N,N'*-bis(2-carboximidoethyl)tartarimide dimethyl ester dihydrochloride (CETD) and *N,N'*-bis(2-carboximidomethyl)tartarimide dimethyl ester dihydrochloride (CMTD). These symmetrical bis(imido esters) contain a *vic*-glycol bond that can be cleaved by treatment with sodium periodate. With the same idea in mind, Lutter et al. (1974) have independently reported cross-linking experiments on the ribosome using a bis azide containing a *vic*-glycol bond. Their paper appeared while we were testing our reagents, and we have adopted their conditions of periodate cleavage. In this paper we describe tests of our reagents with a simple oligomeric protein, fructose bisphosphate aldolase, and cross-linking experiments on the pyruvate dehydrogenase multienzyme complex of *E. coli* with our periodate-cleavable imido esters and with dimethyl suberimidate. A brief account of some of these experiments has been given elsewhere (Coggins, 1975).

Materials and Methods

Enzyme and Reagents. Pyruvate dehydrogenase multienzyme complex from a pyruvate dehydrogenase constitutive mutant of *E. coli* K12 was purified by the method of Reed and Mukherjee (1969). The mutant organism was kindly provided by Professor H. L. Kornberg. Rabbit muscle aldolase (EC 4.1.2.13) was purchased from Boehringer, Ealing, London.

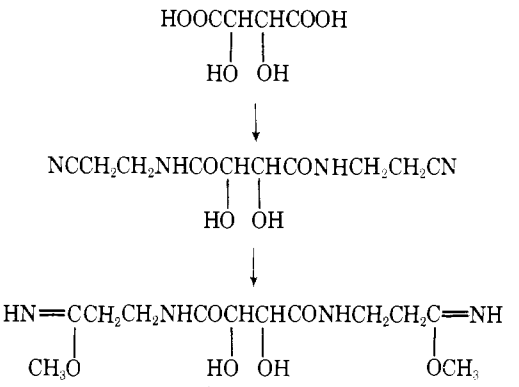
Analar (+)-tartaric acid was obtained from Hopkin and Williams, Romford, Essex; analar dimethylformamide, analar sodium periodate, dicyclohexylcarbodiimide, Dowex 50W-X8, and Amberlite 1RA 400 from B.D.H., Poole, Dorset; 1,6-dicyanohexane from Aldrich, Wembley, Middlesex; and 3-aminopropionitrile from K and K Laboratories, Plainview, N.Y. All other reagents were of analytical reagent grade. Methanol was dried over anhydrous Na_2SO_4, ether was dried over anhydrous $MgSO_4$ and *N*-ethylmorpholine was distilled before use.

Dimethyl suberimidate dihydrochloride was synthesized by the method of Davies and Stark (1970).

N,N'-Bis(2-carboximidoethyl)tartarimide Dimethyl Ester Dihydrochloride and N,N'-Bis(2-carboximidomethyl)tartarimide Dimethyl Ester Dihydrochloride. These reagents were synthesized using the reaction route outlined in Scheme I.

(*a*) *N,N'-Bis(2-cyanoethyl)tartaramide.* A solution of (+)-tartaric acid (3.0 g; 20 mmol) in dimethylformamide (20 ml) was mixed with a solution of 2-aminopropionitrile (2.8 g; 40 mmol) in dimethylformamide (20 ml), and immediately a solution of dicyclohexylcarbodiimide (8.2 g; 40 mmol) in dimethylformamide (10 ml) was added. The mixture was stirred for 16 h at 20 °C, and water (100 ml) was then added. After the mixture was stirred for a further 0.1 h, the dicyclohexylurea was filtered off and the solution was evaporated under reduced pressure at 40 °C. The oily product was extracted with water

Scheme I



(200 ml) and the aqueous extract concentrated to 50 ml by evaporation. This solution was stirred for 0.2 h with Dowex 50W-X8 (30 ml; H^+ form) and then for 0.2 h with Amberlite IRA 400 (30 ml; Cl^- form). The final aqueous solution was evaporated and the resulting oil gave crystals on trituration with dry methanol. Recrystallization from methanol–ether gave 0.5 g of white crystalline product, mp 168–171 °C. The 100 MHz NMR spectrum with trifluoroacetic acid as solvent had three broad peaks at δ 2.89, 3.86, and 5.10, with areas in the ratio of 2.0:2.0:0.9, respectively. On addition of D_2O, the peaks at higher field split into triplets. The ir spectrum (KBr disk) had amide bands at 6.00 μ and 6.48 μ and a nitrile band at 4.45 μ. TLC on silica gel G (unactivated) showed a single spot of R_f 0.43 with 1-butanol/acetic acid/water (4:1:1, v/v) as solvent and R_f 0.24 with chloroform/methanol (9:1, v/v) as solvent. Spots were detected by either the chlorine/starch/KI method (Rydon and Smith, 1952) or the Schiff/periodate method (Fairbanks et al., 1971).

(*b*) *N,N'-Bis(2-carboximidoethyl)tartarimide Dimethyl Ester Dihydrochloride.* An ice-cold suspension of *N,N'*-bis(2-cyanoethyl)tartarimide (0.025 g; 0.1 mmol) in dry methanol (5 ml) was saturated with dry HCl gas and the solution kept at 0 °C for 1 h. Cold dry ether (40 ml) was then added, and after the solution was left standing for 0.5 h at 0 °C the solid product was isolated by decantation and dried under vacuum at 20 °C. The product was hygroscopic and was weighed out for immediate use in cross-linking experiments.

(*c*) *N,N'-Bis(cyanomethyl)tartaramide.* This reagent was synthesized from tartaric acid and aminoacetonitrile hydrochloride by the same procedure as that described above for *N,N'*-bis(2-cyanoethyl)tartaramide. Starting from (+)-tartaric acid (20 mmol), the yield was 1.32 g (29%), mp 185–188 °C. The NMR spectrum in trifluoroacetic acid had two peaks at δ 4.47 (doublet) and 5.13; the peak ratio was 2.0:0.95. The ir spectrum (KBr disk) had amide bands at 6.05 and 6.55 μ and a nitrile band at 4.37 μ. TLC on silica gel G showed a single spot of R_f 0.54 with 1-butanol/acetic acid/water (4:1:1, v/v) as solvent and R_f 0.11 with chloroform/methanol (9:1, v/v) as solvent.

(*d*) *N,N'-Bis(carboximidomethyl)tartarimide Dimethyl Ester Dihydrochloride.* This compound was prepared from *N,N'*-bis(cyanomethyl)tartaramide by using procedure b above.

Yields of the tartaramides were low in these syntheses for two reasons. First, there was incomplete coupling and the work-up was designed to remove half-coupled product containing a free carboxyl group and unreacted starting material. Secondly, in the case of the tartaramide derived from 3-aminopropionitrile, an intramolecular cyclization also contributed to low yields. In one experiment the amount of lactone formed

was great enough to be detected in the crude product by the presence in the ir spectrum of a lactone band at 5.48 μ. This lactone was removed by raising the pH of an aqueous solution of the sample to 12.0 with 1 M NaOH. After 5 min at pH 12 and 20 °C, the desired material was recovered by the ion-exchange and evaporation treatment described. Protection of the *vic*-glycol during the carbodiimide coupling should be effective in raising yields, but this has not yet been investigated.

Cross-Linking Reactions. Protein solutions were exhaustively dialyzed against 0.1 M *N*-ethylmorpholine–acetic acid buffer, pH 8.4. Stock solutions (0.5 M) of cross-linking reagents in a solvent consisting of equal volumes of 1 M buffer and 1 M NaOH were prepared immediately before use. Cross-linking reactions were carried out at 20 °C, with a final protein concentration of 1 mg/ml, estimated spectrophotometrically (Reed and Mukherjee, 1969; Gibbons and Perham, 1970). The concentration of dimethyl suberimidate used was 0.02 M and that of the cleavable cross-linkers was 0.04 M. The final buffer concentration was 0.1 M and if necessary the pH was adjusted to 8.4. The reactions were terminated by adding a few drops of 3 M sodium acetate buffer, pH 5.0, and the protein solutions were then dialyzed against 5 mM potassium phosphate buffer, pH 7.0, overnight at 2 °C.

Sodium Dodecyl Sulfate Gel Electrophoresis. Samples of protein were run in 5% (w/v) polyacrylamide gels containing 0.1% sodium dodecyl sulfate (Shapiro and Maizel, 1969) and the gels calibrated to estimate molecular weights as described previously (Perham and Thomas, 1971). For two-dimensional gels, the first dimension of electrophoresis was carried out in tubes (7.5 cm × 0.5 cm). Periodate cleavage of cross-linked species was effected by soaking the tube gels in 0.02 M phosphate buffer, pH 7.5, containing 0.1% sodium dodecyl sulfate and 0.015 M sodium periodate for 4 h at 20 °C, essentially as described by Lutter et al. (1974). The tube gel was then embedded along the top edge of the slab gel (14 × 14 cm) and sealed in place with a fresh layer of 5% (w/v) polyacrylamide gel before the second dimension of electrophoresis was carried out.

Gels were fixed and stained with Coomassie brilliant blue as described previously (Perham and Thomas, 1971). Tube gels to which equal amounts of protein had been applied were scanned using a Joyce-Loebl Chromoscan densitometer. For a given protein, the relationship between the amount of protein on the gel and the color after staining was assumed to be linear (Vogel et al., 1972).

Resolution of Pyruvate Dehydrogenase Multienzyme Complex. The method used was that of Harrison (1975). Pyruvate dehydrogenase multienzyme complex (200 mg) was dissolved in 6 ml of 20 mM potassium phosphate buffer, pH 7.0, containing EDTA (1 mg/ml) and 0.02% (w/v) sodium azide, at 2 °C. The apparent pH was adjusted to 9.95 with ethanolamine–water (1:1, v/v) and the solution was left on ice for 2 h. The sample was then applied to a column of Sepharose 6B (110 cm × 3.5 cm), equilibrated with the same buffer at the same pH and temperature (2 °C). The flow rate was 30 ml/h, and 9-ml fractions were collected. Protein in the effluent was located by reading the absorbance of samples at 280, 260, and 450 nm. The front peak (unretarded) contains the E2–E3 subcomplex and the retarded peak consists of E1.

The pooled fractions containing each peak were dialyzed against 20 mM potassium phosphate–EDTA–azide buffer, pH 7.0, as before, at 2 °C, and then concentrated to approximately one-tenth the volume by ultrafiltration. The proteins were stored at 2 °C in the same buffer.

The E2–E3 subcomplex was resolved further into the lipoyl transacetylase (E2) and lipoamide dehydrogenase (E3) components by using essentially the method of Koike et al. (1963) except that hydroxylapatite was substituted for calcium phosphate gel cellulose and the urea concentration was raised from 4 to 6 M. The resolved proteins were stored at 2 °C in 20 mM potassium phosphate–EDTA–azide buffer, pH 7.0, as above.

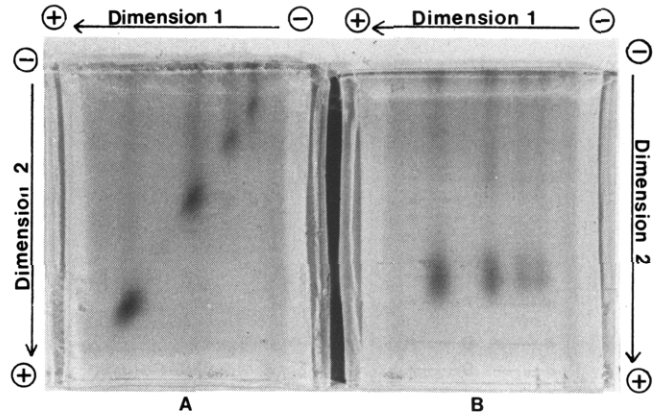


FIGURE 1: Two-dimensional sodium dodecyl sulfate–gel electrophoresis of aldolase cross-linked by treatment with *N,N'*-bis(2-carboximidoethyl)tartarimide dimethyl ester dihydrochloride for 2 h. Slab A, cross-linked aldolase without periodate treatment; slab B, cross-linked aldolase treated with sodium periodate. For other details, see text.

Results

Cross-linking of Aldolase with Dimethyl Suberimidate and with Cleavable Bis(imido esters). When aldolase was treated with dimethyl suberimidate for 1 h and analyzed by sodium dodecyl sulfate gel electrophoresis, four bands were seen with estimated molecular weights of 40 000, 80 000, 115 000, and 150 000. These are the four bands to be expected from the aldolase tetramer (subunit molecular weight of 40 000) as described by Davies and Stark (1970). When aldolase was treated with CETD for 2 h and analyzed by sodium dodecyl sulfate gel electrophoresis, the same four species were observed, and these formed a good diagonal pattern in the two-dimensional separation system (Figure 1A). They can unequivocally be identified as the monomeric, dimeric, trimeric, and tetrameric forms of the aldolase subunit.

If the tube gel used for the first dimension of separation of protein chains cross-linked with CETD was soaked in sodium periodate before the second dimension of electrophoresis was carried out, the diagonal pattern was destroyed and all species reverted to the monomeric form (Figure 1B). Similar results were obtained with aldolase cross-linked by treatment with CMTD. Diagonal patterns obtained with proteins cross-linked by using dimethyl suberimidate were unaffected by the periodate treatment. This was convincing evidence that the periodate treatment acted to break the cleavable cross-links and that the gel electrophoretic systems developed for use with dimethyl suberimidate would serve for analyzing the products of cross-linking with CETD and CMTD.

Cross-linking of Intact Pyruvate Dehydrogenase Multienzyme Complex with Dimethyl Suberimidate. The effect of treating intact pyruvate dehydrogenase complex with dimethyl suberimidate is shown in Figure 2. Samples were removed after various times and analyzed by sodium dodecyl sulfate-gel electrophoresis. The gels were heavily loaded with protein to ensure that minor species would be visible. In the untreated control sample, bands 6, 7, and 9 can be identified as E1, E2, and E3, with subunit molecular weights of ca.

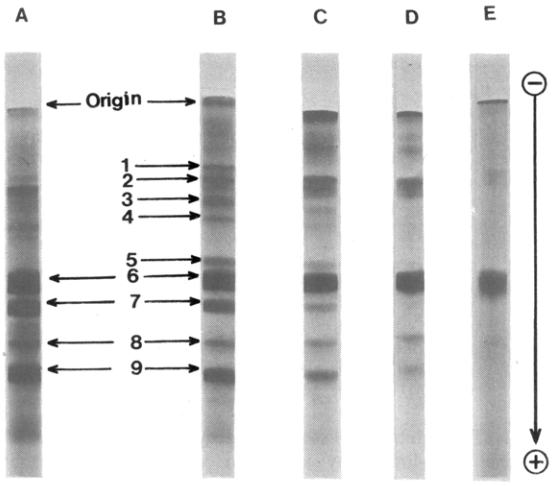


FIGURE 2: Sodium dodecyl sulfate–gel electrophoresis of pyruvate dehydrogenase multienzyme complex after treatment with dimethyl suberimidate. Gel A, untreated complex; gel B, complex treated for 40 min; gel C, complex treated for 100 min; gel D, complex treated for 200 min; gel E, complex treated for 24 h. For other details, see text.

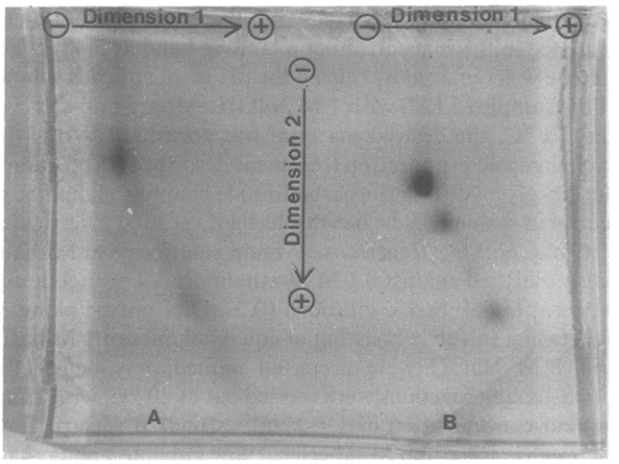


FIGURE 4: Two-dimensional sodium dodecyl sulfate–gel electrophoresis of pyruvate dehydrogenase multienzyme complex cross-linked by treatment with *N,N'*-bis(2-carboximidoethyl)tartarimide dimethyl ester dihydrochloride for 4 h. Side A, cross-linked complex treated with sodium periodate; side B, control (unmodified) complex treated with sodium periodate. For other details, see text.

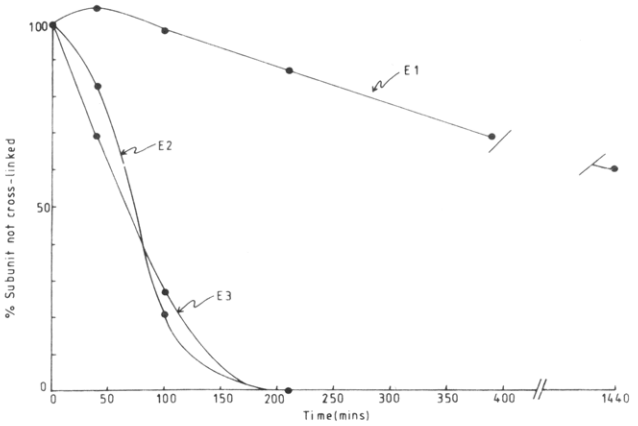


FIGURE 3: Cross-linking of the components (E1, E2, E3) of the pyruvate dehydrogenase multienzyme complex with dimethyl suberimidate, estimated by quantitative scanning of sodium dodecyl sulfate–polyacrylamide gels after staining with Coomassie brilliant blue. Samples of the cross-linking reaction mixture were removed at various time intervals and analyzed by sodium dodecyl sulfate–gel electrophoresis as shown in Figure 2. For other details, see text.

100 000, 80 000, and 56 000, respectively (Perham and Thomas, 1971). The faint bands 2 and 8 have apparent molecular weights of 200 000 and 65 000, respectively, the latter material frequently being observed as a trace component or contaminant of the multienzyme complex purified in this laboratory. Bands 1, 3, 4, and 5 have estimated molecular weights of ca. 230 000, 180 000, 160 000, and 115 000, respectively.

The most striking feature of the time course of the reaction is the persistence of band 6, attributed tentatively to the subunit of E1, while the bands derived from E2 and E3 disappear and heavily cross-linked material appears at the top of the gels where it hardly penetrates. Quantitative scanning of the gels shows that, to a first approximation, subunits of E2 and E3 disappear at the same rate and that a situation can be reached in the intact complex in which E2 and E3 are highly cross-linked to each other but with virtually no cross-links involving E1 (Figure 3).

From the estimated molecular weights, band 5 (Figure 2) can be identified tentatively as the cross-linked dimer of E3. This species appears early in the cross-linking reaction and gradually disappears as cross-links are made with E2. The identities of bands 1, 3, and 4 are less certain and this exemplifies the difficulty in interpreting patterns of cross-links in proteins with complex quaternary structures that contain several different types of polypeptide chain. However, the analysis is simplified a little if we exclude, as we apparently can, contributions from E1. For example, band 1 could be a tetrameric form of E3 or a trimer of E2; band 3 is possibly a trimer form of E3, whereas band 4 is likely to be a dimer of E2. Band 2 has the properties of a trace dimer form of E1 but since it, and the trace component of mol wt 65 000, persist long into the time course of the cross-linking reaction, we have disregarded them at this stage of identifying cross-linked species.

The possibility that bands 1–5 of Figure 2 represent intermolecularly cross-linked species is made extremely unlikely by two facts. One is that when the experiments were carried out at different protein concentrations (0.5–4.0 mg/ml), no significant change in cross-linking pattern was apparent. Secondly, at all such protein concentrations, the molar concentration of the multienzyme complex is very low indeed.

Cross-linking of Pyruvate Dehydrogenase Multienzyme Complex with Cleavable Bis(imido esters). Although the resolution of the gels shown in Figure 2 is good, enabling bands 5 and 6 to be identified with some confidence as the cross-linked dimer of E3 and the monomeric subunit of E1, it was essential to be able to show unequivocally that band 6 was not a cross-linked species. Pyruvate dehydrogenase multienzyme complex was therefore treated with CETD for 4 h. As with dimethyl suberimidate, the principal product observed in sodium dodecyl sulfate gel electrophoresis was a species with an estimated molecular weight of 100 000. Weak bands corresponding with bands 1–5 of Figure 2 were also seen. The cross-linked complex was therefore examined by diagonal sodium dodecyl sulfate gel electrophoresis. In Figure 4B is shown a control sample of multienzyme complex, the major protein components (E1, E2, E3) falling on the expected diagonal. The complex that had been cross-linked and then subjected to periodate cleavage before the second dimension of electrophoresis (Figure 4A) gave only one major spot, which corresponded in position with the uncross-linked monomer of E1. Faint spots corresponding with the uncross-linked mono-

mers of E2 and E3 were also visible, which helped to establish the position of the diagonal, but no off-diagonal proteins were present. The fainter cross-linked species observed in the first dimension of electrophoresis were in too low a yield to be visible as off-diagonal spots after the second dimension. We therefore conclude that band 6 of Figure 2 is the uncross-linked monomer of E1 and that it is not masking another cross-linked species. This result strengthens the conclusion that band 5 is the dimeric form of E3.

In Figure 4B, the intensity of the spot E1 relative to spots E2 and E3 is stronger than it is in the tube gel of untreated multienzyme complex shown in Figure 2. This is apparently due to differences in the efficiency of transfer of the components from the tube gel to the slab gel in the two-dimensional separation and was frequently observed.

Cross-Linking of Pyruvate Decarboxylase Component (E1) and Lipoamide Dehydrogenase Component (E3) with Dimethyl Suberimidate. The resolved pyruvate decarboxylase component (E1) of the pyruvate dehydrogenase multienzyme complex was treated with dimethyl suberimidate and examined by sodium dodecyl sulfate gel electrophoresis. The results are shown in Figure 5. Even after 24 h reaction, very little cross-linking was observed, the weak band of cross-linked protein running with an estimated molecular weight of ca. 200 000. At early stages in the cross-linking reaction, the band of cross-linked protein shows a triplet substructure (gels B and C of Figure 5). The same triplet band can be seen very faintly in the control sample of untreated E1 (gel A of Figure 5) and in the crosslinking of the intact complex with dimethyl suberimidate (gel C of Figure 2). The reason for the existence of this triplet structure is at present unclear, but it should be pointed out that the loading of the gels is very heavy and that, particularly in the untreated control sample, the triplet banding may be artifactual.

On the other hand, cross-linking of the resolved lipoamide dehydrogenase component (E3) with dimethyl suberimidate for 1 h caused two bands of estimated molecular weight 56 000 and 115 000 to be seen on sodium dodecyl sulfate–gel electrophoresis, consistent with the accepted dimeric structure of this component of the pyruvate dehydrogenase multienzyme complex observed in free solution (Koike et al., 1963).

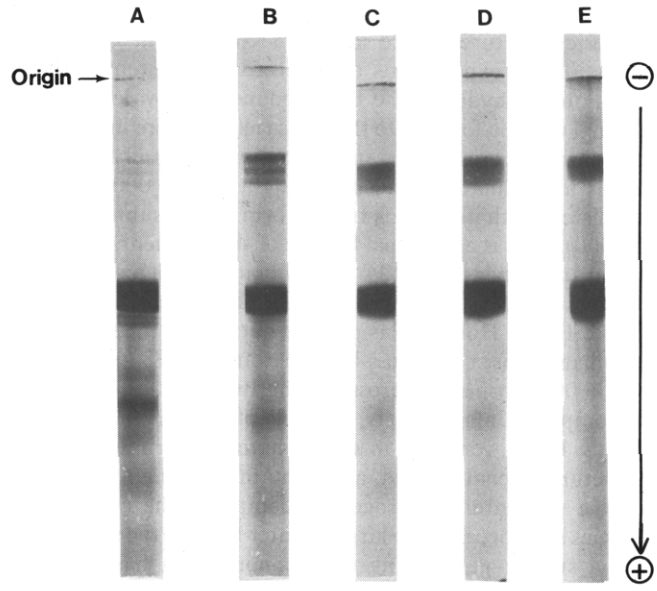


FIGURE 5: Sodium dodecyl sulfate–gel electrophoresis of the pyruvate decarboxylase (E1) component of the pyruvate dehydrogenase multienzyme complex after treatment with dimethyl suberimidate. Gel A, untreated E1; gel B, E1 treated for 40 min; gel C, E1 treated for 100 min; gel D, E1 treated for 200 min; gel E, E1 treated for 24 h. For other details, see text.

Discussion

The inability to cross-link the subunit of the pyruvate decarboxylase component, either to itself or to the lipoyl transacetylase and lipoamide dehydrogenase components of the pyruvate dehydrogenase multienzyme complex, with dimethyl suberimidate (Figure 2) or the periodate-cleavable bis(imido ester) CETD (Figure 4) is surprising, since there is no doubt that the three types of polypeptide chain are physically associated in the complex (Reed and Oliver, 1968; Reed, 1974). The use of the cleavable cross-linking agent was essential in proving that the protein species of apparent molecular weight 100 000 observed in sodium dodecyl sulfate–gel analysis of the cross-linked complex was the E1 subunit. These observations therefore serve as a warning that cross-linking experiments can falsely indicate a lack of subunit contact in certain circumstances, although the use of longer cross-linking reagents might yet reveal the subunit contacts of E1. The inability to form intersubunit cross-links with any of the bis(imido esters) described in this paper is presumably caused by the absence of suitable amino groups from the neighborhood (within the span length of the reagents, say 1–1.5 nm) of the subunit interfaces. It is not due to a general lack of reactivity of the amino groups in protein chains of E1 because comparable experiments with the monofunctional imido ester, methyl acetimidate, show that most, if not all, of the amino groups in E1 are accessible to modification in the intact complex (D. L. Bates, J. Coggins, and R. N. Perham, unpublished work). Neither is it due to dissociation of E1 from the complex under the cross-linking conditions as judged by ultracentrifugation or gel filtration through Sepharose columns, during which experiments E1 clearly remains attached (E. A. Hooper and R. N. Perham, unpublished work). Indeed, the pH has to be raised above 9.0 before E1 begins to dissociate from the complex (Koike et al., 1963). There is little doubt therefore that cross-links are formed, but they must be within and not between subunits.

On the other hand, the E2 and E3 components are evidently closely associated and permit intersubunit cross-links to be formed with great facility (Figure 2). The E2 component is known to form a structural core around which the other two components are arranged (Reed and Oliver, 1968), but the symmetry is still a matter of debate. It is probable that, like the lipoyl transsuccinylase from the corresponding 2-oxoglutarate dehydrogenase complex (DeRosier et al., 1971), the lipoyl transacetylase has octahedral (432) symmetry (Reed, 1974), which demands the existence of 24 packing units (Reed, 1974; Perham, 1975), presumably polypeptide chains, although it is theoretically possible, in somewhat unlikely special circumstances, to make up the structure from 12 polypeptide chains (Perham and Thomas, 1971). However, an alternative structure comprising 16 transacetylase chains has also been proposed (Vogel et al., 1972). This structure is incompatible with octahedral symmetry (Reed, 1974; Perham, 1975), but since the symmetry of the transacetylase core has not been unequivocally determined, a firm decision about the subunit structure cannot be taken at this stage.

The cross-linking experiments reported here give some hopeful clues. Band 5 in Figure 2 is clearly the cross-linked dimer from the lipoamide dehydrogenase, E3, which has a subunit molecular weight of 56 000. The resolved and separate enzyme yields the same dimer band when cross-linked with

dimethyl suberimidate, which is compatible with its accepted dimeric structure in free solution (Koike et al., 1963). The cross-linking experiments are therefore consistent with the lipoamide dehydrogenase being bound noncovalently in its normal dimeric form on to the transacetylase core in the intact complex. The cross-linked species comprising band 1 of Figure 2 has an estimated molecular weight of 230 000, which is consistent with its being a trimer form of E2 (subunit mol wt 80 000). This is a principal feature of the octahedral model of the transacetylase, the 24 chains being arranged in trimer clusters at the eight corners of a cube (Reed and Oliver, 1968; Reed, 1974). However, as pointed out above, the same band could also consist of a cross-linked tetramer of E3 (subunit mol wt 56 000) formed by cross-linking two cross-linked dimers in complex. Unfortunately, the yield of intermediates such as bands 1–5 is very low in cross-linking experiments with a structure as complicated, and containing as many subunits, as the pyruvate dehydrogenase complex. The cross-linked species soon became too large to enter far into the sodium dodecyl sulfate–gels during electrophoresis. It has therefore proved impracticable, so far, to identify the intermediates by using diagonal gel electrophoresis and the cleavable cross-linking reagents. However, we hope that similar experiments with the simpler transacetylase core will enable a choice to be made between the conflicting structural models.

The inability to cross-link the resolved pyruvate decarboxylase component was also surprising, since the enzyme has been reported to be a dimer of mol wt 200 000 in free solution at neutral pH (Vogel and Henning, 1971; Eley et al., 1972). However, the cross-linking experiments were carried out at pH 8.4, and a possible reconciliation of these results is that free E1 might be largely monomeric at this pH. Support for this idea comes from a preliminary analysis in the ultracentrifuge of the pH dependence of the state of aggregation of E1 (Hooper and Perham, unpublished work) in which we find that the enzyme is apparently monomeric at pH of 9 and above, and is principally dimeric at pHs of 7 and below. Moreover, Dennert and Eaker (1970) have reported the coexistence of monomeric and dimeric forms of the enzyme at pH 7, the dimer having dissociated to the monomer at pH 9.5. Unfortunately, owing to the pH dependence of their reaction with amino groups, imido esters cannot be used to effect cross-linking in proteins at pHs much below 8, and the sedimentation behaviour of E1 will therefore need to be studied in more detail to test these ideas. Cross-linking reagents that are effective at pHs below 7 would also be of value.

The reactions of aldolase and the pyruvate dehydrogenase multienzyme complex with the periodate-cleavable bis(imido esters) clearly demonstrate the utility of these reagents. The periodate treatment between the two steps of a diagonal gel electrophoretic separation breaks the cross-links with no apparent side reactions and, in all other respects, the reagents resemble their noncleavable counterparts. The identification of the subunit of E1 as the principal band remaining in sodium dodecyl sulfate–gel separations of multienzyme complex extensively cross-linked with dimethyl suberimidate (Figure 2) was confirmed by the comparable experiment carried out with CETD (Figure 4). It may sometimes be necessary to make use of cleavable cross-linkers with different chain lengths in the analysis of subunit contacts, and it is easy to envisage that periodate-cleavable cross-linkers longer than the two we describe here could be synthesized.

Under the conditions of pH, etc., described in this paper, no cross-linking of aldolase could be achieved by using the bisazides described by Lutter et al. (1974), the pH needed to secure cross-linking being substantially higher than that used for bis(imido esters) (J. R. Coggins, unpublished work). The latter reagents therefore offer the advantage of applicability nearer to neutral pH. Many valuable results have been obtained by using cross-linking reagents containing disulfide bridges that can be cleaved by reduction at the appropriate moment (Sun et al., 1974; Wang and Richards, 1974; Thomas and Kornberg, 1975). However, as we have mentioned earlier, these reagents have some inherent disadvantages. The periodate-cleavable bis(imido esters) do not suffer from these drawbacks, and we hope that they will prove useful in a wide variety of applications.

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The Mechanism of the Aminoacylation of Transfer Ribonucleic Acid: Enzyme•Product Dissociation Is Not Rate Limiting†

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ABSTRACT: It has been proposed that the rate-limiting step in the synthesis of aminoacyl-tRNA is the rate at which the product dissociates from the enzyme. The experimental evidence supporting this hypothesis comes from work at low pH and low temperature (although the reaction has been argued to have the same mechanism under physiological conditions). We have reexamined the binding assay by which M. Yarus and P. Berg (1969) (*J. Mol. Biol. 42,* 171-189) measured the k_d for dissociation of Enz•(Ile-tRNA). We find that when overall reaction and dissociation are measured under identical conditions the two rates are not the same. Moreover, while an increase in ionic strength greatly stimulates dissociation, the same increased ionic strength slows aminoacylation. Spermine accelerates overall aminoacylation without affecting dissociation. Because any change in a rate-limiting step must, by definition, cause a parallel change in the overall reaction, these observations prove that under these conditions the synthesis of Ile-tRNA is not limited by the rate of dissociation of Enz•(Ile-tRNA). Entirely similar observations were made for the dissociation of Enz•(Val-tRNA) and the overall synthesis of Val-tRNA at 0 °C, pH 5.0. In addition, valine enzyme isolated by nitrocellulose filtration during the course of an aminoacylation was shown not to be saturated with recently synthesized Val-tRNA. The enzyme was in equilibrium with uncharged substrate tRNA and with product Val-tRNA. E. W. Eldred and P. R. Schimmel ((1972) *Biochemistry 11,* 17-23) report that the formation of Ile-tRNA proceeds at two rates: (a) $k = 2 \times 10^{-2}$ s^{-1} until the enzyme is saturated with the first mole of product, and (b) $k = 2 \times 10^{-3}$ s^{-1} for subsequent cycles. We did not observe this behavior at any pH or temperature with four different amino acid:tRNA ligases. Because aminoacylation proceeds more rapidly than "dissociation" under some conditions, we believe that the binding assay measures not only enzyme•product dissociation but also other slower reactions such as aggregation or disaggregation of Enz•(AA-tRNA). In conjunction with recent studies from other laboratories, this work makes it unlikely that enzyme•product dissociation is the rate-limiting step in the synthesis of aminoacyl-tRNA either at low temperature and pH or under more nearly physiological conditions. From the effect of salt, it would appear that the rate of aminoacylation of tRNA is largely limited by the rate or extent of formation of Enz•(tRNA) (Loftfield, R. B., and Eigner, E. A. (1967), *J. Biol. Chem. 242,* 5355-5359). Using the binding assay of M. Yarus ((1972) *Biochemistry 11,* 2050-2060), we find the K_{ass} for Enz•(Ile-tRNA) varies linearly with the Debye-Hückel function at ionic strengths of 0.1-0.4 from 10^8 to 10^6.

It has been proposed that the rate-limiting step in the synthesis of several aminoacyl-tRNAs (AA-tRNA[1]) is the separation of the product from the enzyme (Yarus and Berg, 1969; Hélène et al., 1971; Eldred and Schimmel, 1972, 1973). If a particular step is rate limiting, a change in rate of that step should change the overall reaction in a parallel way.

It seemed probable that raising the ionic strength would accelerate dissociation (Pingoud et al., 1973; Krauss et al., 1973; Loftfield and Eigner, 1967); therefore, we sought to determine, under Yarus and Berg's binding assay conditions, the effect of ionic strength on the overall reaction. In general under other conditions, it is known that higher ionic strengths slow aminoacylation reactions (Loftfield and Eigner, 1967; Taglang, et al., 1970; Smith, 1969; Holten and Jacobson, 1969; Yarus, 1972; Loftfield, 1972). The only reported exception to this generalization involves the tRNA and enzymes derived from halophilic bacteria (Griffiths and Bayley, 1969). Similarly, it was likely that spermine would accelerate the overall reaction (Igarashi et al., 1971; Pastuszyn and Loftfield, 1972), so we examined the effect of spermine on dissociation.

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[1] Abbreviations used are: $tRNA^{AA}$, unesterified transfer ribonucleic acid specific for a particular amino acid; $AA\text{-}tRNA^{A}$, transfer ribonucleic acid esterified with its specific amino acid; Enz, free enzyme (amino acid:tRNA ligase specific for a particular amino acid (EC 6.1.1)); AA~AMP aminoacyl adenylate; Enz•($tRNA^{Ile}$), Enz•(Val-tRNA), Enz•(AA~AMP), etc., ligase bound to $tRNA^{Ile}$, to Val-tRNA, to aminoacyl adenylate etc., respectively; EDTA, (ethylenedinitrilo)tetraacetic acid; DEAE, diethylaminoethyl; GSH, reduced glutathione.