Quaternary Structure of the Pyruvate Dehydrogenase Multienzyme Complex of *Bacillus stearothermophilus* Studied by a New Reversible Cross-Linking Procedure with Bis(imidoesters)†

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ABSTRACT: The bis(amidine) cross-links formed between protein subunits by treating them with bis(imidoesters) were found to be rapidly broken by exposing the cross-linked proteins to methylamine buffers containing the aprotic solvent acetonitrile. This cleavage step could be introduced between the two dimensions of a diagonal gel electrophoretic separation of cross-linked proteins to facilitate identification of the contributors to a cross-linked species. Tests with the tetrameric enzyme glyceraldehyde-3-phosphate dehydrogenase demonstrated the simplicity and effectiveness of the technique. When the pyruvate dehydrogenase multienzyme complex from *Bacillus stearothermophilus* was treated with a range of bis(imidoesters), from dimethyl succinimidate to dimethyl suberimidate, the most informative set of products was obtained with dimethyl glutarimidate. The longer bis(imidoesters) caused too extensive cross-linking of the enzyme subunits, although the β chain of the pyruvate decarboxylase component always appeared to be the most resistant. Almost all the cross-linked species up to pentamers of the lipoate acetyltransferase polypeptide chain (apparent M_r approximately 280 000) were identified by means of the diagonal gel electrophoretic procedure after cleavage of the cross-links. The introduction of the methylamine cleavage step enables the bis(imidoester) for such experiments to be selected purely for the efficacity of its cross-linking reaction with the protein and dispenses with the need to incorporate a specially cleavable bond in the reagent.

Cross-linking reactions with bis(imidoesters) have been widely used to study the quaternary structure of proteins in solution since the introduction of dimethyl suberimidate for this purpose (Davies & Stark, 1970). Bis(imidoesters) have been devised with specially tailored interior bonds that can be cleaved at will to help overcome the difficulties in identifying protein chains that contribute to a cross-linked species. Such bonds include the disulfide bridge (Sun et al., 1974; Wang & Richards, 1974; Thomas & Kornberg, 1975; Jue et al., 1978) and the *vic*-glycol bond (Coggins et al., 1976, 1977). These reagents have proved of great value (Peters & Richards, 1977; Thomas, 1978; Perham et al., 1980), but their usefulness has been circumscribed by the additional complication in their synthesis and the restricted choice of chain length between the terminal imidoester groups.

Following the demonstration that treatment with methylamine buffers rapidly removes acetimidoyl groups from proteins without deleterious side effects (DuBois et al., 1981), we reasoned that it should be possible to adapt this as a general cleavage reaction for proteins cross-linked with any bis(imidoester). The range of the cross-linking approach would thus be greatly extended by the removal of the limitations inherent in the conventional use of cleavable bis(imidoesters).

In the present paper we show that methylamine buffers can be used to break bis(amidine) cross-links between proteins as part of the analysis of quarternary structure by polyacrylamide gel electrophoresis, and we describe an application of the new technique to the pyruvate dehydrogenase multienzyme complex of *Bacillus stearothermophilus*. This unusually large enzyme complex consists of multiple copies of four different types of polypeptide chain (E1α, apparent M_r 42 000; E1β, apparent M_r 36 000; E2, apparent M_r 57 000; E3, apparent M_r 54 000) and is based on a structural core of the E2 component comprising 60 E2 polypeptide chains arranged with icosahedral symmetry (Henderson et al., 1979; Henderson & Perham, 1980). It may be expected therefore to constitute a suitably demanding subject for analysis by cross-linking with bis(imidoesters).

Materials and Methods

Chemicals. Acetonitrile and methylamine solution [25%/30% (w/v) in water] were purchased from BDH Chemicals Ltd., Poole, Dorset, U.K. Dimethyl suberimidate dihydrochloride was from Pierce and Warriner (U.K.) Ltd., Cheshire, U.K. Bis(imidoesters) of shorter chain length were synthesized from the corresponding nitriles by the method of Davies & Stark (1970).

Enzymes. Glyceraldehyde-3-phosphate dehydrogenase (from rabbit muscle) was obtained from BCL, Sussex, U.K. The pyruvate dehydrogenase complex was purified from *B. stearothermophilus* by the method of Henderson & Perham (1980).

Chemical Cross-Linking of Glyceraldehyde-3-phosphate Dehydrogenase. Glyceraldehyde-3-phosphate dehydrogenase (0.5 mg/mL) was incubated at 23 °C with 11 mM dimethyl suberimidate in 0.1 M *N*-ethylmorpholineacetic acid buffer, pH 8.2. After 30 min, the reaction was arrested by adding an equal volume of ice-cold trichloroacetic acid [50% (w/v)]. The precipitated protein was collected by centrifugation, washed twice with acetone, and dried under vacuum.

Chemical Cross-Linking of Pyruvate Dehydrogenase Complex from B. stearothermophilus. The bis(imidoesters) of different chain length used in exploring the subunit structure of the pyruvate dehydrogenase complex from *B. stearothermophilus* were dimethyl succinimidate, dimethyl glutarimidate, dimethyl adipimidate, dimethyl pimelimidate, and dimethyl suberimidate. These reagents have, respectively, two to six methylene groups between the imidoester functions of the molecule.

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Samples of pyruvate dehydrogenase complex (1 mg/mL) were incubated at 23 °C with 10 mM bis(imidoester) in 0.1 M *N*-ethylmorpholineacetic acid buffer, pH 8.5. An untreated sample of enzyme served as a control. After 30 and 60 min, samples (20 μL) were withdrawn from each incubation, mixed with 5 μL of glacial acetic acid to arrest the reaction, and then freeze-dried.

Separation of Cross-Linked Polypeptide Chains and Cleavage of Cross-Links. Duplicate samples of cross-linked protein were analyzed by polyacrylamide gel electrophoresis in the presence of 0.1% (w/v) $NaDodSO_4$[1] by the tube gel system [5% T, 2.6% C (Hjerten, 1962)] with phosphate buffers (Shapiro et al., 1967; Hale & Perham, 1979). One gel of the pair was stained with Coomassie Brilliant Blue to determine the cross-linking pattern; the second untreated gel was frozen rapidly at −70 °C, sealed in polythene film, and could be stored at −20 °C until required. While still frozen, the gel was sliced longitudinally. Half of the gel was maintained at −20 °C, and the other half was treated as follows:

The gel was incubated at 37 °C in a solution (15 mL) of 1.8–2.15 M methylamine, pH 11.5, in 75% (v/v) acetonitrile. This solution was prepared by adding 2.5 mL of concentrated HCl to 20 mL of aqueous methylamine solution [25–30% (w/v)] and was adjusted to pH 11.5 if necessary. The pH was measured on a sample of solution diluted 10-fold with distilled water. A volume of methylamine solution, pH 11.5, was mixed with 3 volumes of acetonitrile to produce a working solution. The gel, which became white and opaque during the treatment, was incubated with three changes of the methylamine–acetonitrile solution for a total period of 3 h. After this time, the treated gel and the untreated gel that had been kept frozen were incubated at 37 °C for a total time of 1 h in four changes of 15 mL of 0.1 M Tris-HCl, pH 6.8, containing 0.1% (w/v) $NaDodSO_4$. This buffer is present in the stacking gel used in the second dimension of electrophoresis (see below). Each gel became transparent once more.

Electrophoresis in the Second Dimension. Electrophoresis of treated and untreated gels in the second dimension was performed in slab gels with the $NaDodSO_4$–Tris–glycine buffer system (Laemmli, 1970). Mercaptoacetic acid (5 mM) was included in the electrode buffers. The disc gels, prepared as described above, were placed approximately 0.5 cm above the resolving gel (11.5% T, 1% C). The stacking gel solution (5% T, 2.5% C) was poured around the disc gels and allowed to set. After electrophoresis, the gels were stained with 0.1% (w/v) Coomassie Brilliant Blue R in methanol–acetic acid–water [5:1:5 (v/v)] and destained in 7% (v/v) acetic acid. Selected gels were stained with the more sensitive silver method (Morrisey, 1981), which was applied subsequent to or as an alternative to staining with Coomassie Brilliant Blue.

Results

Glyceraldehyde-3-phosphate Dehydrogenase. The general method of cleaving bis(amidine) cross-links in gels was developed with glyceraldehyde-3-phosphate dehydrogenase as a model protein. The tetrameric enzyme (M_r 144 000) was cross-linked with dimethyl suberimidate, and the product of the reaction was analyzed by $NaDodSO_4$–polyacrylamide gel electrophoresis. Four main *n*-mers, ranging from monomer (M_r 36 000) to tetramer were observed, with small amounts of higher aggregates also present (Figure 1a). Incubation of the gels at 37 °C in 2 M methylamine–HCl, pH 11.5, in 75% (v/v) acetonitrile produced a quantitative cleavage of the cross-links within 3 h, thereby regenerating the monomeric polypeptide chains, as demonstrated by electrophoresis of the proteins in a second dimension (Figure 1b).

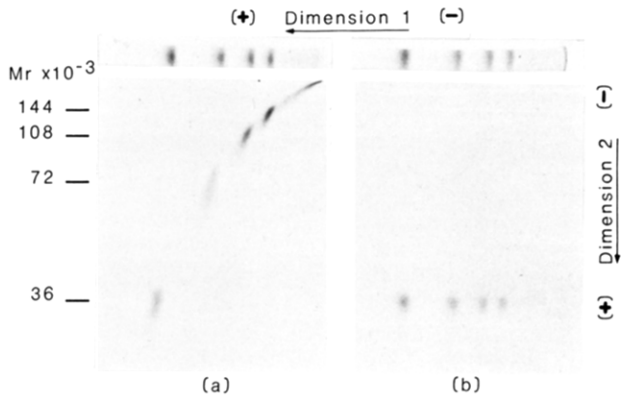

FIGURE 1: Cleavage of cross-links in dimethyl suberimidate treated glyceraldehyde-3-phosphate dehydrogenase. Glyceraldehyde-3-phosphate dehydrogenase was cross-linked with dimethyl suberimidate as described in the text. The cross-linked polypeptide chains were separated by $NaDodSO_4$–polyacrylamide gel electrophoresis in phosphate-buffered tube gels (5% T, 2.6% C). The tube gel was sliced longitudinally into halves. Half was kept frozen, and the other half was treated with 2 M methylamine, pH 11.5, in 75% (v/v) acetonitrile for 3 h at 37 °C. The two halves of the gel were then prepared for electrophoresis in the second dimension as described in the text. After the second electrophoresis, in a Tris–glycine-buffered slab gel system (11.5% T, 1% C), the gels were stained with Coomassie Brilliant Blue. (a) Diagonal gel electrophoresis of cross-linked protein. (b) Diagonal gel electrophoresis of cross-linked protein with intermediate treatment with methylamine to cleave the cross-links. A marker gel of the cross-linked enzyme is shown above each slab.

We found that cleavage of cross-links was incomplete in 3 h when acetonitrile was omitted or when its concentration was reduced to 50% or below. Under these conditions, increasing the methylamine concentration to 3.4 M, pH 11.5, had little effect on the yield of monomeric protein. The inclusion of 5 mM mercaptoacetic acid in the electrode buffers during electrophoresis in the second dimension was found to be beneficial in preventing the formation of spurious cross-links in the form of disulfide bridges between polypeptide chains.

Pyruvate Dehydrogenase Complex from B. stearothermophilus. This new method of cleaving bis(amidine) cross-links was applied in a study of the quaternary structure of the pyruvate dehydrogenase complex from *B. stearothermophilus*. Samples of the multienzyme complex were incubated with a range of bis(imidoesters) (10 mM) for 30 min at 23 °C. The products of the reaction were separated by $NaDodSO_4$–polyacrylamide gel electrophoresis (Figure 2). Dimethyl succinimidate effected little, if any, cross-linking of the polypeptide chains. In contrast, the longest cross-linkers of the series (dimethyl pimelimidate and dimethyl suberimidate) induced a rapid polymerization that led mostly to material of high M_r (>500 000) that failed to enter the gel. Dimethyl malonimidate also gave rise to polymers of very high molecular weight although protein species of intermediate size were weakly present. Treatment with dimethyl glutarimidate seemed best suited for further analysis since the cross-linked species, although present in low yield, were mostly included within the gel. Cross-linking the enzyme complex with each bis(imidoester) for 60 min induced more extensive polymerization with the three longest cross-linkers only and gave no more useful information.

The extent of cross-linking with dimethyl glutarimidate was similar over a 2-fold range of reagent concentration (10–20 mM) and a 5-fold range of protein concentration (0.2–1.0

[1] Abbreviations: $NaDodSO_4$, sodium dodecyl sulfate; Tris, tris(hydroxymethyl)aminomethane.

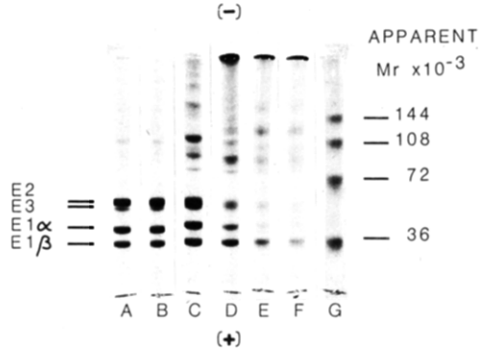

FIGURE 2: Cross-linking of pyruvate dehydrogenase complex from *B. stearothermophilus* with bis(imidoesters) of different chain length. Pyruvate dehydrogenase complex (1 mg/mL) was incubated at pH 8.5 and 23 °C with 10 mM bis(imidoester) for 30 min. The reaction was arrested by acidification, and the polypeptide chains (10 μg) were separated by $NaDodSO_4$–polyacrylamide gel electrophoresis (5% T, 2.6% C) in phosphate-buffered tube gels. The position of the dye front was marked by insertion of a wire into the gel before staining with Coomassie Brilliant Blue. (Track A) Untreated complex; (track B) complex treated with dimethyl succinimidate; (track C) complex treated with dimethyl glutarimidate; (track D) complex treated with dimethyl adipimidate; (track E) complex treated with dimethyl pimelimidate; (track F) complex treated with dimethyl suberimidate; (track G) glyceraldehyde-3-phosphate dehydrogenase cross-linked with dimethyl suberimidate as approximate molecular weight markers.

mg/mL). The identity of the cross-linked products was difficult to establish with certainty in a single gel system owing to the similar molecular weight to be expected for different species. The poor resolution of the un-cross-linked E2 and E3 bands after the treatment (Figure 2, track C) presented an added problem in assessing whether E3 was cross-linked to any significant degree. Our approach was therefore to establish the identity of the polypeptide chains present in each cross-linked species by cleaving the cross-links and analyzing the cleavage products by $NaDodSO_4$–polyacrylamide gel electrophoresis in a second dimension (Figure 3). Note that, in Tris–glycine slab gels where T > 15%, the electrophoretic mobility of E3 is lower than that of E2 although the former protein has a smaller apparent molecular weight (Henderson & Perham, 1980).

The staining intensity of the protein bands in the slab gel, obtained with Coomassie Brilliant Blue, was insufficient to allow the unambiguous identification of the cleavage products of every cross-linked species. However, by use of the more sensitive silver stain, identical gels were developed to different levels of staining intensity (Figure 3). Several conclusions could be drawn from these gels. The E2 and E3 polypeptide chains were confirmed to be present in the single diffuse protein band (M_r 53 000–56 000) observed in the tube gel of cross-linked enzyme complex where these monomers were unresolved. Monofunctional substitution of E2 with dimethyl glutarimidate clearly affects its electrophoretic mobility in the first dimension. The smallest cross-linked product (M_r 80 000) evidently comprised E1α (M_r 42 000) and E1β (M_r 36 000). The next largest product in significant yield was in fact present in the tube gels as a closely spaced doublet (M_r 88 000–92 000), a feature more readily seen in a 7% polyacrylamide gel (Figure 3c). The cleavage products of the band of smaller molecular weight could be identified as E1α only, whereas the second band comprised E2 (M_r 57 000) and E1β. A faint cross-linked product of M_r 100 000 may be identified, from Figure 3b, as resulting from an E2–E1α cross-link. The major cross-linked product (M_r 110 000–112 000) had at least two components. Figure 3a shows the presence of E2 plus distinctly smaller amounts of E3 slightly offset with respect to the position of the E2 chain. Cross-links must therefore have occurred between two E2 chains, but we were unable to determine whether the E3 component was present as a dimer, as an E2-E3 species, or indeed as a mixture of these two forms. The higher cross-linked species were predominantly polymers of E2 with molecular weight values corresponding to trimers and probably

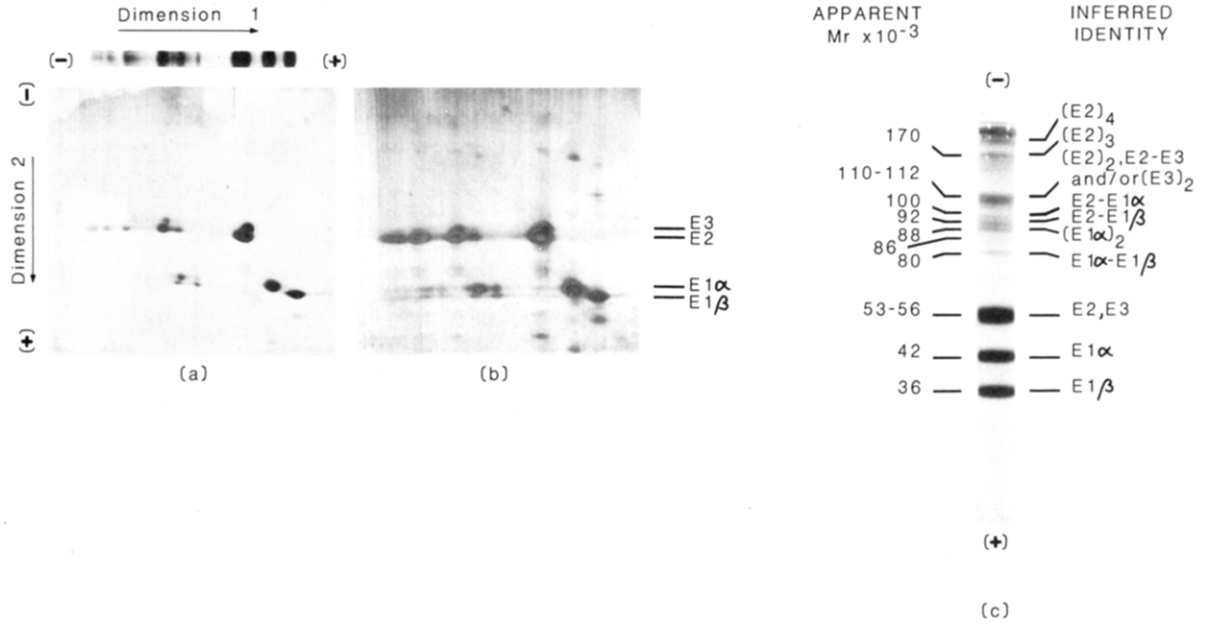

FIGURE 3: Cleavage of cross-links in dimethyl glutarimidate treated pyruvate dehydrogenase complex. Pyruvate dehydrogenase complex was cross-linked with 10 mM dimethyl glutarimidate. The products of the reaction were separated by $NaDodSO_4$–polyacrylamide gel electrophoresis (5% T, 1.6% C) in phosphate-buffered tube gels. The tube gel was sliced in half longitudinally, and each half was treated with methylamine–acetonitrile solution as described in the text. After electrophoresis in the second dimension (11.5% T, 1% C) in the Tris–glycine-buffered system, the two slab gels were stained to differing intensities with silver stain (a and b). A marker tube gel of the cross-linked complex stained with Coomassie Brilliant Blue is shown above slab a. A sample of cross-linked pyruvate dehydrogenase complex was electrophoresed in a $NaDodSO_4$–polyacrylamide tube gel of higher (7% T, 2.6% C) acrylamide concentration, resulting in improved resolution of some of the protein bands (c). This gel was stained with Coomassie Brilliant Blue. The inferred identity of the polypeptide chains present in each cross-linked species is indicated on that gel.

tetramers and pentamers. Figure 3b shows that E1α, E1β, and E3 chains are also present, in much smaller amounts, in the polymers of high molecular weight. On the basis of these results, we conclude that the identities of the cross-linked products are as shown in Figure 3c. We were unable to identify the nature of the band of M_r 86 000 owing to its poor yield.

Of ancillary interest was the discovery (Figure 3a,b) that the cleavage reaction led to some polypeptide chain degradation. The same effect was noted when un-cross-linked samples of the protein were similarly treated (data not shown). The amount of degradation and polymerization observed here with E2/E3, E1α, and E1β was clearly very small and was more readily detected in the silver-stained gels than in Coomassie-stained gels because of their higher sensitivity. We do not foresee such degradation causing a serious problem in the study of protein quaternary structure by this method.

Discussion

It is clear from the experiments with glyceraldehyde-3-phosphate dehydrogenase (Figure 1) and with the pyruvate dehydrogenase multienzyme complex of *B. stearothermophilus* (Figure 3) that cleavage of the cross-links formed by treatment of proteins with bis(imidoesters) can be achieved simply and effectively by exposing the cross-linked proteins to methylamine buffers. The inclusion of acetonitrile [75% (v/v)] in the methylamine solution was found to be essential if complete cleavage was to be attained in a reasonable incubation time (3 h). Presumably the acetonitrile is acting as an aprotic solvent promoting the nucleophilicity of the methylamine in the transamidination reaction [cf. Dubois et al. (1981)]. The method of reversible cross-linking we have described permits any bis(imidoester) to be used as reagent in the analysis of quaternary structure by two-dimensional polyacrylamide gel electrophoresis and does away with the need to include a special target bond for cleavage in the reagent itself.

This new approach is exemplified by the experiments with the pyruvate dehydrogenase complex of *B. stearothermophilus*. Thus, it was found that with a range of bis(imidoesters) tested, dimethyl glutarimidate gave the most informative set of cross-linked products (Figure 2), the detailed analysis of which was made possible by reversing the cross-links with methylamine and submitting the proteins to a second dimension of polyacrylamide gel electrophoresis. There was extensive cross-linking of all enzyme subunits with the longer bis(imidoesters), although the E1β polypeptide chain always appeared to be the most resistant (Figure 2). This echoes the result with the pyruvate dehydrogenase complex of *Escherichia coli*, where treatment with dimethyl suberimidate failed to form cross-links between the E1 polypeptide chains and the E2 or E3 components (Coggins et al., 1976). The reason for this is still obscure since the E1 chains are known to be bound tightly but noncovalently to the E2 core (Reed, 1974). A simple explanation could be the absence of suitably placed amino groups from the neighborhood of the subunit interfaces, and it emphasizes the need to interpret cross-linking results with caution (Coggins et al., 1976).

Many cross-linked species could be identified in the analysis of *B. stearothermophilus* pyruvate dehydrogenase complex treated with dimethyl glutarimidate (Figure 3). The existence of E1α–E1β, $(E1\alpha)_2$, E2–E1β, E2–E1α, and E2–E2 species was demonstrated unequivocally, and the identification of $(E2)_3$, and $(E2)_4$ is hardly less certain. Since the structural core of the complex consists of 60 E2 polypeptide chains arranged as a pentagonal dodecahedron (Henderson et al., 1979), the opportunities for generating higher oligomers or polymers of the E2 chain are manifold. As these oligomers mount in size, they rapidly become impossible to resolve by sodium dodecyl sulfate–polyacrylamide gel electrophoresis. The same limitation in applying the cross-linking technique to large protein assemblies built up from a few different types of polypeptide chain was noted in earlier experiments with the *E. coli* pyruvate dehydrogenase complex (Coggins et al., 1976). The E1 component of the pyruvate dehydrogenase complex from mammalian mitochondria is thought to exist as an $(E1\alpha)_2(E1\beta)_2$ tetramer in free solution (Reed, 1974), and our cross-linking results with the highly analogous pyruvate dehydrogenase complex from *B. stearothermophilus* are consistent with a close association of E1α, E1β, and E2 subunits in the assembled enzyme.

The lipoamide dehydrogenase (E3) component of the *B. stearothermophilus* pyruvate dehydrogenase complex, like that of *E. coli*, is a dimer in free solution (Henderson, 1979), but no $(E3)_2$ species was positively identified in our cross-linking experiments. The reasons for this are probably 2-fold. First, the small amount of E3 component in the starting complex (Figure 2, track A) means that if it were distributed among a number of cross-linked species (e.g., oligomers of E2), it would effectively disappear. Second, the similarity in molecular weight of the E2 and E3 chains observed in sodium dodecyl sulfate–polyacrylamide gel electrophoresis made it difficult to resolve a mixture of E3–E3, E3–E2, and E2–E2 species. The major cross-linked product, apparent M_r 110 000–112 000, shown in Figure 3, was found to contain E2 and E3 polypeptide chains by polyacrylamide gel electrophoresis in a second dimension after cleavage of the cross-links. We could infer that, in addition to a main E2–E2 species, there must also have been present E2–E3 and/or E3–E3 species. The latter would be expected to occur since it was readily detected after cross-linking *E. coli* pyruvate dehydrogenase complex with dimethyl suberimidate (Coggins et al., 1976). Certainly, there is no reason to suppose that E3 does not exist as a dimer in the pyruvate dehydrogenase complex of *B. stearothermophilus*.

The effectiveness of bis(imidoesters) for cross-linking proteins has been widely appreciated. We hope that our simple procedure for reversing these cross-links without deleterious effect on the primary structure of the polypeptide chains will widen still further their use in a variety of protein chemical studies.

Acknowledgments

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References

Coggins, J. R., Hooper, E. A., & Perham, R. N. (1976) *Biochemistry 15*, 2527–2533.

Coggins, J. R., Lumsden, J., & Malcolm, A. D. B. (1977) *Biochemistry 16*, 1111–1116.

Davies, G. E., & Stark, G. R. (1970) *Proc. Natl. Acad. Sci. U.S.A. 66*, 651–656.

DuBois, G. C., Robinson, E. A., Inman, J. K., Perham, R. N., & Appella, E. (1981) *Biochem. J. 199*, 335–340.

Hale, G., & Perham, R. N. (1979) *Eur. J. Biochem. 94*, 119–126.

Henderson, C. E. (1979) Ph.D. Thesis, University of Cambridge.

Henderson, C. E., & Perham, R. N. (1980) *Biochem. J. 189*, 161–172.

Henderson, C. E., Perham, R. N., & Finch, J. T. (1979) *Cell (Cambridge, Mass.) 17*, 85–93.

Hjerten, S. (1962) *Arch. Biochem. Biophys., Suppl. 1*, 147.
Jue, R., Lambert, J. M., Pierce, L. R., & Traut, R. R. (1978) *Biochemistry 17*, 5399–5406.
Laemmli, U. K. (1970) *Nature (London) 227*, 680–685.
Morrisey, J. H. (1981) *Anal. Biochem. 117*, 307–310.
Perham, R. N., Appella, E., DuBois, G. C., & Inman, J. K. (1980) in *Methods of Peptide and Protein Sequence Analysis* (Birr, C., Ed.) pp 345–348, Elsevier/North-Holland, Amsterdam.
Peters, R., & Richards, F. M. (1977) *Annu. Rev. Biochem. 46*, 525–551.
Reed, L. J. (1974) *Acc. Chem. Res. 7*, 40–46.
Shapiro, A. L., Vinuela, E., & Maizel, J. V. (1967) *Biochem. Biophys. Res. Commun. 28*, 815–820.
Sun, T.-T., Bollen, A., Kahan, L., & Traut, R. R. (1974) *Biochemistry 13*, 2334–2340.
Thomas, J. O. (1978) in *Techniques in Protein and Enzyme Biochemistry* (Kornberg, H. L., Metcalfe, J. C., Northcote, D. H., Pogson, C. I., & Tipton, K. F., Eds.) Vol. B106, pp 1–22, Elsevier/North-Holland, Amsterdam.
Thomas, J. O., & Kornberg, R. D. (1975) *Proc. Natl. Acad. Sci. U.S.A. 72*, 2626–2630.
Wang, K., & Richards, F. M. (1974) *J. Biol. Chem. 249*, 8005–8018.

Adenosine 3′,5′-Cyclic Monophosphate Protein Kinase from Bovine Brain: Inactivation of the Catalytic Subunit and Holoenzyme by 7-Chloro-4-nitro-2,1,3-benzoxadiazole†

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ABSTRACT: NBD-Cl (7-chloro-4-nitro-2,1,3-benzoxadiazole) inactivates the catalytic subunit of adenosine 3′,5′-cyclic monophosphate (cAMP) dependent protein kinase isolated from bovine brain by covalent modification. The reaction follows pseudo-first-order kinetics. The pseudo-first-order rate constant (k_{app}) shows a hyperbolic dependence on NBD-Cl concentration, suggesting formation of a reversible complex before covalent modification with a dissociation constant (K_i) of 150 μM. MgATP, MgADP, and adenosine protect against inactivation 75, 60, and 50%, respectively, whereas ATP, ADP, or Mg^{2+} alone does not protect. This process was competitive in nature (K_a for adenosine 44 μM). Protein substrates (histone IIa, 2 mg/mL; Leu-Arg-Arg-Ala-Ser-Leu-Gly, 1 mM) do not protect against inactivation; histone actually accelerates the rate of inactivation. Inactivation is associated with modification of 2.1 ± 0.15 mol of cysteine/mol of catalytic subunit, determined spectrophotometrically and radioisotopically. Activity can be restored by treating inactivated enzyme with 2-mercaptoethanol. MgATP protects one cysteine on the average from modification while protecting against inactivation. In the absence of cAMP, type I and II regulatory subunits from bovine skeletal muscle and type II regulatory subunit from bovine brain slow modification of the catalytic subunit by NBD-Cl. In the presence of 0.6 mM cAMP the brain holoenzyme is inactivated slower than the free catalytic subunit, but type I and II holoenzymes from skeletal muscle are inactivated faster than the free catalytic subunit. This suggests that the regulatory and catalytic subunits communicate even in the presence of saturating concentrations of cAMP. The effect of brain regulatory subunit on the reactivity of sulfhydryls in the catalytic subunit is opposite that of the skeletal muscle regulatory subunits. The latter's effect on sulfhydryl reactivity is similar to that observed by Armstrong and Kaiser for the bovine heart enzyme [Armstrong, R. N., & Kaiser, E. T. (1978) *Biochemistry 17*, 2840], suggesting differences in the interactions of the regulatory and catalytic subunits in the enzymes from these different tissues.

Adenosine 3′,5′-cyclic monophosphate (cAMP) dependent protein kinase (ATP:protein phosphotransferase, EC 2.7.1.37) catalyzes the phosphorylation of polypeptidic serine and threonine residues. The enzyme consists of dissimilar regulatory (R) and catalytic (C) subunits.[1] Until recently the enzyme was reported to be activated by binding 2 mol of cAMP and dissociating into free C subunits and dimeric $R_2(cAMP)_2$ complexes (Gill & Garen, 1970; Tao et al., 1970; Rosen & Erlichman, 1975; Beavo et al., 1975). Recent studies, however, indicate that each regulatory dimer can bind 4 mol of cAMP (Corbin et al., 1978; Weber et al., 1979), yielding the following activation scheme:

$$R_2C_2 + 4cAMP \rightleftharpoons R_2(cAMP)_4 + 2C$$

Work in this and other laboratories is aimed at identifying amino acids present at the active site of the C subunit as well as studying the mechanism of inactivation of the C subunit by regulatory proteins.

Possible amino acid residues important to enzyme function have been assessed by chemical modification studies. Characterization of ethoxyformic anhydride inhibition of C subunit (Witt & Roskoski, 1975a), for example, suggests that a tyrosine residue occurs in the active site. Chemical modification of cysteine residues also inhibits enzyme activity (Sugden et al., 1976; Peters et al., 1977; Armstrong & Kaiser, 1978).

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[1] Abbreviations: R, regulatory subunit of cAMP-dependent protein kinase; C, catalytic subunit of cAMP-dependent protein kinase; FSBA, 5′-[*p*-(fluorosulfonyl)benzoyl]adenosine; NBD-Cl, 7-chloro-4-nitro-2,1,3-benzoxadiazole; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); Mops, 3-(*N*-morpholino)propanesulfonic acid; EDTA, ethylenediaminetetraacetic acid; Ser-peptide, Leu-Arg-Arg-Ala-Ser-Leu-Gly; DEAE, diethylaminoethyl; $NaDodSO_4$, sodium dodecyl sulfate.

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