A Method for the Separation of Hybrids of Chromatographically Identical Oligomeric Proteins. Use of 3,4,5,6-Tetrahydrophthaloyl Groups as a Reversible "Chromatographic Handle"†

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ABSTRACT: Hybridization experiments with variants of an oligomeric protein often provide important information regarding subunit structure, function, and interactions. In some systems, however, the variants are so similar electrophoretically and chromatographically that purification of individual hybrids is not feasible. Therefore a method was developed for preparing hybrids by using 3,4,5,6-tetrahydrophthalic anhydride as a reversible acylating agent for protein amino groups. The technique involved acylating about 30% of the amino groups at pH 8 to give a derivative with a markedly altered net charge, formation of the hybrid set with unmodified and modified species, separation of the individual components by ion-exchange chromatography, and finally removal of the tetrahydrophthaloyl groups from the desired hybrid by incubation for about 1 day at pH 6 and room temperature. Experiments with model compounds and two enzymes showed that the anhydride was specific for amino groups. The extent of modification of proteins was measured by the spectral change at 250 nm, the loss of free amino groups, and the change in electrophoretic mobility of the polypeptide chains in polyacrylamide gels containing 8 *M* urea. Deacylation of modified, inactive aldolase and the catalytic subunit of aspartate transcarbamylase led to the restoration of the enzyme activity and electrophoretic mobility of the unmodified proteins. Both *intra*- and *inter*-subunit hybrids of aspartate transcarbamylase were prepared and isolated by using the tetrahydrophthaloyl groups as a reversible "chromatographic handle". Prior to deacylation the *inter*-subunit hybrid containing one acylated and one native catalytic subunit (and negative regulatory subunits) exhibited no homotropic cooperativity and after deacylation the characteristic allosteric properties of the enzyme were regained. Similarly the ligand-promoted conformational changes associated with the allosteric transition were restored upon deacylation of the *intra*-subunit hybrid containing one acylated and two native chains in each catalytic subunit. Criteria are described which must be satisfied if a reversible "chromatographic handle" is to be effective in hybridization experiments and it is shown that, despite some heterogeneity in its reaction with protein amino groups, 3,4,5,6-tetrahydrophthalic anhydride shows considerable promise for studies of oligomeric proteins.

The number and properties of the hybrid species formed from structural variants of an oligomeric protein have provided valuable knowledge about the quaternary structure, function, and subunit interactions of many enzymes (Markert, 1963, 1968; Kaplan, 1968; Penhoet et al., 1966, 1967; Meighen et al., 1970, 1971; Penhoet and Rutter, 1971; Gibbons, 1974). For some systems the detection of hybrids and their separation from one another and from the parental molecules are feasible because the parental species differ significantly in their electrophoretic and chromatographic behavior. Often, however, these differences are so small that fractionation of the hybrid set to give the individual components cannot be achieved. These latter systems involving, for example, hybrids from a native enzyme and its inactive variant produced either by a point mutation or by selective modification with a site-specific reagent are frequently of considerable interest. The inability to study such well-defined hybrids prompted us to develop a general method which would permit the separation of hybrids of structurally similar variants which are not readily resolvable by available methods. The technique involves the introduction of a removable "chromatographic handle" into one parental oligomer which significantly alters its net charge, formation of the hybrid set between the unmodified and modified species, fractionation of the mixture by ion-exchange chromatography or electrophoresis, and finally removal of the modifying groups from the purified hybrids by a change in pH sufficiently small that the structure of the oligomeric hybrids is not disrupted.

Cyclic anhydrides of γ dicarboxylic acids (such as succinic and maleic anhydrides) have proved to be valuable reagents for the production of reasonably homogeneous, stable derivatives of oligomeric enzymes having quaternary structures similar to the unmodified proteins. Moreover, these derivatives in conjunction with the native proteins have been used successfully to generate hybrids which could be separated readily by ion-exchange chromatography (Meighen and Schachman, 1970; Meighen et al., 1970; Shifrin and Grochowski, 1972; Gibbons, 1974). Although not totally specific these anhydrides do react preferentially with amino groups and thereby for each group reacted cause a net change of -2 in the charge on the protein. The derivatives produced by maleic and related anhydrides can be decomposed readily at low pH by an intramolecular reaction involving anchimeric assistance of the free carboxyl group of the maleamic acid moiety (Leach and Lindley, 1953;

† From the Department of Molecular Biology and the Virus Laboratory, University of California, Berkeley, California 94720. *Received July 28, 1975.* This research was supported in part by Public Health Service Research Grant GM 12159 from the National Institute of General Medical Sciences and Grant GB 32812X from the National Science Foundation.

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Butler et al., 1969). In this process, the mechanism of which is discussed in detail by Kirby and Lancaster (1972), the original amino groups are regenerated. The rate of deacylation is dependent upon the extent of protonation of the maleamic acid and is remarkably sensitive to the pattern of substitution of the carbon–carbon double bond (Dixon and Perham, 1968; Kirby and Lancaster, 1972).

In order to exploit the phenomenon of acylation and deacylation as a means of reversibly introducing charged groups for hybridization of oligomeric proteins we needed a derivative which satisfied two criteria. First, it must be sufficiently stable at neutral or slightly alkaline pH to permit dissociation of the oligomeric protein and reconstitution to form hybrids followed by chromatographic manipulations for the separation of the various species. Second, after the desired modified hybrid is obtained it should be capable of deacylation at a lower pH which is sufficiently close to neutrality that the quaternary structure of the oligomers is not affected during the removal of the acyl groups. Both maleic and 2-methylmaleic (citraconic) anhydrides have been shown to react reversibly with amino groups in proteins (Dixon and Perham, 1968; Butler et al., 1969) but the deacylation rates at pH values which do not lead to dissociation of many oligomeric proteins are too slow to be of use for this application. However, the data of Kirby and Lancaster (1972) indicated that 3,4,5,6-tetrahydrophthalic anhydride (H_4Pht anhydride)[1] might be a suitable reagent satisfying the above requirements. This paper shows the utility of H_4Pht anhydride for the introduction of H_4Phtoyl groups which serve as a "chromatographic handle" for hybridization experiments. Experiments with model compounds are described and the successful applications of the anhydride for the preparation of hybrids of aspartate transcarbamylase are illustrated.[2] Despite the heterogeneity of modification of the amino groups on different chains in an oligomeric enzyme, the derivatives are still sufficiently homogeneous to permit successful fractionation of the members in the hybrid sets formed between native and N-acylated proteins. Deacylation at a pH about 6 is sufficiently rapid and complete to restore not only enzyme activity in inactivated proteins but also the characteristic kinetic properties of allosteric enzymes.

Materials and Methods

Materials. N^{α}-Acetyl-L-lysinamide and N^{α}-acetylcysteine were the products of the Cyclo Chemical Corp. Butylamine was obtained from Calbiochem and trinitrobenzenesulfonic acid (TNBS) and glycine from Eastman Organic Chemicals. Rabbit muscle aldolase and the reagents used in the assay for its enzymic activity (NADH, triosephosphate isomerase, and α-glycerolphosphate dehydrogenase) were obtained from C. F. Boehringer und Sohne, G.M.B.H., Mannheim, Germany. Aspartate transcarbamylase (ATCase) was prepared according to Gerhart and Holoubek (1967); the catalytic subunit (C) and regulatory subunit (R) from ATCase were isolated by the method of Kirschner (1971). Succinylation of C was performed as described by Nagel and Schachman (1975) with succinic anhydride obtained from Eastman Kodak. Reagent grade 1,4-dioxane (J. T. Baker) which had been redistilled just prior to use was employed as a solvent for both succinic anhydride and for H_4Pht anhydride (Aldrich Chemical Co.).

Synthesis of Model Compounds. The N-H_4Phtoyl derivatives of glycine and N^{α}-acetyl-L-lysinamide were made by the addition of 1.0 equiv of H_4Pht anhydride (0.5 *M* in 1,4-dioxane) to 0.1 *M* stirred solutions of the amino compounds at 23°; the pH was maintained about 9 by the addition of 2 *M* NaOH. H_4Phtoyl-butylamine was synthesized by adding 1.0 equiv of butylamine dropwise to 0.6 *M* H_4Pht anhydride dissolved in dioxane. The resulting white precipitate was dissolved by heating to 60° and the product crystallized. Assays for free amino groups by the method of Habeeb (1966) with TNBS showed less than 5% of free amino groups for all of the acylated products. After incubating the derivatives at pH 2 for a few hours, more than 95% of the free amino groups were regenerated.

Kinetics of Deacylation of Model Compounds. Deacylation of the H_4Phtoyl derivatives was performed by incubating 2 m*M* solutions at 22° in the following buffer systems: (a) 50 m*M* citric acid–K^+ at pH 2.5–3.5; (b) 50 m*M* acetic acid–K^+ at pH 3.5–5.5; and (c) 50 m*M* phosphoric acid–K^+ at pH 5.5–7.5. Deacylation was stopped at appropriate times by dilution of the samples into 50 m*M* borate buffer at pH 9, and the extent of reaction was determined by the TNBS assay for free amino groups (Habeeb, 1966).

Protein Concentrations. Spectrophotometric measurements of the absorbance at 280 nm were used for the determination of protein concentrations based on the known extinction coefficients. Since the reaction with H_4Pht anhydride had little effect on the absorbance at 280 nm, no corrections were made for the contribution of the H_4Phtoyl groups to the absorbance. Also the increase in molecular weight due to the H_4Phtoyl groups was neglected in determinations of the protein concentration.

Enzyme Activity. Aldolase was assayed by the method of Gibbons and Perham (1974) and ATCase by the method of Porter et al. (1969) or by the colorimetric procedure of Prescott and Jones (1969).

Protein Thiol Groups. Ellman's reagent (Ellman, 1959) in the presence of 5.8 *M* guanidine hydrochloride was used for the determination of thiol groups as described by Gibbons and Perham (1974). The extinction coefficient used for the thionitrobenzoate anion was corrected by a factor of 7% to allow for the spectral change produced by guanidine hydrochloride (I. Gibbons, unpublished observation).

Acylation of Proteins with H_4Pht Anhydride. Protein solutions at 5 mg/ml in 50 m*M* phosphate buffer at pH 8 containing 2 m*M* EDTA and 2 m*M* β-mercaptoethanol were stirred at 0° and H_4Pht anhydride (0.5 *M* in 1,4-dioxane) was added and the solution was stirred until the pH was constant. Generally it was not necessary to adjust the pH of the solution which decreased by less than 0.5 pH unit with the largest amounts of anhydride. After about 30 min the protein solution was dialyzed against 50 m*M* Tris-HCl–2 m*M* EDTA–2 m*M* β-mercaptoethanol at pH 8.25 to remove H_4Pht acid. The modified proteins were stored at 4° and pH 8.5.

Deacylation of H_4Phtoyl Proteins. Deacylation was accomplished at room temperature by incubating the modi-

[1] Abbreviations used are: H_4Pht, 3,4,5,6-tetrahydrophthalic; H_4Phtoyl, 3,4,5,6-tetrahydrophthaloyl; TNBS, 2,4,6-trinitrobenzenesulfonic acid; ATCase, aspartate transcarbamylase; C, catalytic subunit of ATCase; R, regulatory subunit of ATCase; N (subscript), native subunit; T (subscript), tetrahydrophthaloylated subunit; P (subscript), pyridoxylated subunit; P,T (subscript), pyridoxylated and tetrahydrophthaloylated subunit; n (subscript), native polypeptide chain; t (subscript), tetrahydrophthaloylated polypeptide chain.

[2] The application of H_4Pht anhydride for the preparation of a hybrid of ATCase containing one active and one chemically inactivated catalytic subunit and three native regulatory subunits has been described elsewhere (Gibbons et al., 1974).

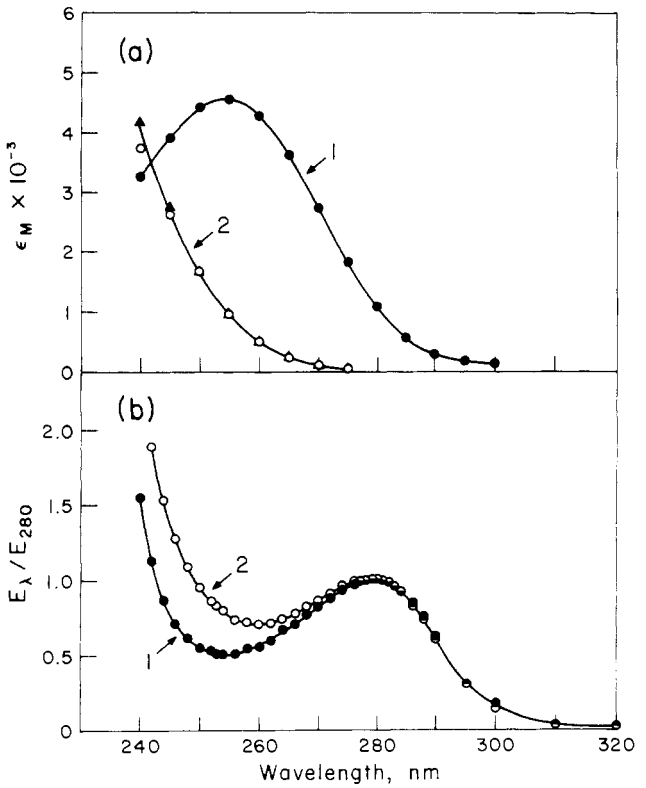

FIGURE 1: Absorption spectra of H_4Pht anhydride and its derivatives. (a) The plots represent the molar extinction coefficient as a function of wavelength. Curve 1 designated by ● shows data for H_4Pht anhydride in dioxane. Curve 2 gives the data for the acylated derivative of butylamine (○) in 10 m*M* $Na_2B_4O_7$ at pH 9 and for the acylated derivative (5.5 H_4Phtoyl groups per chain) of the catalytic subunit of ATCase (▲) corrected for the protein absorbance. It was assumed for the correction that the absorbance of the acylated protein at 280 nm was the same as that of the unmodified protein. The number of H_4Phtoyl groups per protein molecule was determined by the assay with TNBS. (b) The absorbance normalized to 280 nm is plotted as a function of wavelength for the unmodified catalytic subunit (curve 1, ●) and for the acylated derivative (curve 2, ○). Both spectra were measured on protein solutions in 10 m*M* $Na_2B_4O_7$ at pH 9.

fied protein (0.1–0.5 mg/ml) for 1–2 days in 50 m*M* phosphate buffer at pH 6.05. The buffer contained 2 m*M* EDTA and 2 m*M* β-mercaptoethanol. For ATCase-like molecules the pH of the solution was adjusted to a value slightly above 6.5 before the protein solution was concentrated by ultrafiltration over Amicon PM-10 membranes or by dialysis against 3.6 *M* ammonium sulfate containing 5 m*M* EDTA and 10 m*M* β-mercaptoethanol. Increasing the pH of the solution was necessary in order to avoid isoelectric precipitation of the protein (Gerhart and Holoubek, 1967).

Hybridization of Native and H_4Phtoyl-Catalytic Subunits and Chains of ATCase. The *inter*-subunit hybrid set was prepared according to Meighen et al. (1970) by mixing C_N and C_T and adding regulatory subunits, R, to give reconstituted ATCase-like molecules. The *intra*-subunit hybrid set was prepared by the following procedure: equal amounts of C_N and C_T in 50 m*M* Tris-Cl–2 m*M* EDTA–2 m*M* β-mercaptoethanol at pH 8.25 were mixed to give a total protein concentration of 5–10 mg/ml. One volume of this mixture, at 0°, was added with stirring to 4 volumes of a solution of 8 *M* urea and 0.1 *M* dithiothreitol in the same buffer. After 15 min the dissociated and denatured protein was diluted rapidly at 0° into 9 volumes of the above buffer. The resulting solution was then dialyzed against two changes of buffer in order to remove the urea. The hybrid, $C_{nnt}C_{nnt}R_3$, was made by adding excess R to a preparation of C_{nnt} made by the *intra*-subunit hybridization procedure. Gel filtration on Sephadex G-200 was used to remove the excess R.

Chromatography of the Hybrid Sets. Individual members of the hybrid set were purified by ion-exchange chromatography on DEAE-Sephadex columns at pH 7.5 according to the procedures of Meighen et al. (1970) for the *inter*-subunit set and Pigiet (1971) for the *intra*-subunit set. Prolonged exposure of the labile H_4Phtoyl derivatives to pH values lower than 8 was avoided by dialyzing the hybrid sets against buffers of pH above 8 with compositions such that the appropriate buffer for chromatography was obtained merely by adding an adjusting solution just prior to the fractionation.

Electrophoresis. Zone electrophoresis was performed on cellulose polyacetate strips (Gelman Sepraphore) in a Microzone electrophoresis cell (Beckman-Spinco) as described by Meighen et al. (1970). Polyacrylamide gel electrophoresis in 5% gels was conducted with the Tris-glycine system of Jovin (1973). Electrophoresis in the presence of 8 *M* urea was performed in 3% polyacrylamide gels (0.15% bisacrylamide) for 2 hr at 2 mA/gel. Samples were applied in 8 *M* urea–10 m*M* β-mercaptoethanol at pH about 8. Gels were stained with Coomassie Brilliant Blue G-250 (Diezel et al., 1972), and scanning of the individual protein bands was performed with a Joyce-Loebl microdensitometer fitted with a red filter.

Sedimentation. Measurements of ligand-promoted changes in sedimentation coefficient ($\Delta s/\bar{s}$) were made according to Gerhart and Schachman (1968) with 40 m*M* phosphate buffer at pH 7, containing 0.2 m*M* EDTA and 2 m*M* β-mercaptoethanol. The pH of the solution was adjusted from 8.5 to 7 just prior to the ultracentrifuge experiments. In this way loss of the H_4Phtoyl groups was minimized (about 4% in the course of a 3-hr experiment).

Results

Reaction of H_4Pht Anhydride with Amino Groups. Reaction of the anhydride with protein amino groups leads to an increase in the absorbance at wavelengths below 270 nm because of the conjugated double bond system of the H_4Phtoyl groups. Curve 1 in Figure 1a shows the absorption spectrum of H_4Pht anhydride in dioxane. The maximum at 254 nm with a molar extinction coefficient of 4.5×10^3 M^{-1} cm^{-1} provides a useful measure of the concentration of the anhydride. When the anhydride reacts with butylamine and the acylated product is examined in aqueous solution at pH 9, only end absorption (curve 2 in Figure 1a) is observed in the near-ultraviolet region of the spectrum. Figure 1b shows that at wavelengths above 280 nm the spectrum of the catalytic subunit of ATCase is hardly affected by acylation (5.5 amino groups modified per polypeptide chain). In contrast, the increase in absorbance of the acylated protein below 270 nm is clearly demonstrated by comparison of curves 1 and 2 in Figure 1b. This difference (normalized per H_4Phtoyl group) is plotted in Figure 1a and it is seen that the data (curve 2) are in excellent agreement with those obtained for the amide formed with butylamine. Thus the increase in absorption at about 250 nm, where most proteins exhibit a minimum in their spectrum, can be used as a measure of acylation of amino groups with H_4Pht anhydride.

Since maleic and other related anhydrides have been

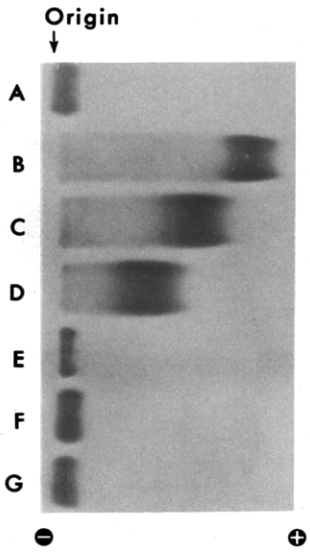

FIGURE 2: Electrophoresis patterns for aldolase reacted with H_4Pht anhydride. All patterns were obtained on cellulose acetate strips as described by Meighen and Schachman (1970). The pattern in A is that of native aldolase and those in B, C, and D represent acylated derivatives formed with molar ratios of H_4Pht anhydride to protein amino groups corresponding respectively to 1.0, 0.5, and 0.25. The patterns in E, F, and G show the deacylated products from B, C, and D, respectively. Deacylation was performed for 6 hr at 26° in 50 m*M* acetate (NH_4^+), 2 m*M* EDTA, and 2 m*M* β-mercaptoethanol at pH 5. The samples were then dialyzed at 4° overnight against 20 m*M* acetate (NH_4^+) buffer (pH 5.5) containing 2 m*M* EDTA and 2 m*M* β-mercaptoethanol.

found to alkylate cysteinyl side chains in proteins (Gibbons and Perham, 1970, 1974), it seemed important to determine whether H_4Pht anhydride also reacted with sulfhydryl groups. Accordingly the reaction of a protein like aldolase (at 5 mg/ml) with H_4Pht anhydride was simulated by incubating *N*-acetylcysteine at a concentration of 1 m*M* with 2.5 equiv of the anhydride (this corresponds to two times the ratio of anhydride used below for acylation of proteins). No loss of sulfhydryl groups was observed as measured by the assay with Ellman's reagent.

Deacylation of Model Compounds. The kinetics of removal of the H_4Phtoyl groups of N^{ϵ}-H_4Phtoyl-N^{α}-acetyl-L-lysinamide were examined at room temperature at varying pH values. From pH 2.5 to about 6.5 the reaction was first order. Below pH 3 the rate was independent of pH with a half-time of hydrolysis of 8.6 min (at 22°) and above pH 5 the half-time of the reaction increased about tenfold for each unit increase in pH as observed by Kirby and Lancaster (1972) with other amides. The data yielded an apparent p*K* of 4.3 for the free carboxyl group and half-times of hydrolysis of 370 min and 60 hr at pH 6 and 7, respectively. The deacylation of α-H_4Phtoyl-glycine was much faster with a half-time of 1 hr at pH 6.2.

Reversible Acylation of Aldolase. As seen in Figure 2, treatment of rabbit muscle adolase with H_4Pht anhydride produced reasonably homogeneous derivatives with electrophoretic mobilities substantially different from that of the native enzyme (A). Samples B, C, and D were obtained in acylation experiments in which the molar ratios of H_4Pht anhydride to total amino groups in the protein were 1.0, 0.5, and 0.25, respectively. Although sample C was largely inactivated (less than 10% of the activity of the native enzyme) as a result of the acylation, the protein was still in the tetrameric form with a sedimentation coefficient of 7.3 S. Upon

Table I: Loss of Enzymic Activity upon Acylation of Catalytic Subunit and Regeneration upon Deacylation.

Derivative No.	Ratio of H_4Pht Anhydride to Amino Groups (mol/mol)	Extent of Acylation[a] (mol/mol)	Specific Activity of Derivative[b] (%)	Regeneration of Enzymic Activity[c] (%)
1	0.12		61	
2	0.23		31	
3	0.35		18	
4	0.45	4.6	7	93
5	0.60	5.5	2	66

[a] Number of amino groups acylated per catalytic polypeptide chain as measured by the loss of free amino groups which react with TNBS. [b] Enzymic activity measured by the colorimetric assay and expressed as percent of unmodified catalytic subunit. [c] Enzymic activity of deacylated samples expressed as percent of native subunit which was treated in the same manner except for the reaction with H_4Pht anhydride. Deacylation was performed by incubation of the derivative (0.25 mg/ml) for 20 hr at 23° in 50 m*M* phosphate buffer at pH 6 containing 2 m*M* EDTA and 2 m*M* β-mercaptoethanol. The sample was then dialyzed in a similar buffer at pH 7.

deacylation of this derivative at pH 5, almost 100% of the original enzymic activity was restored. Moreover, as seen by the electrophoretic patterns designated E, F, and G in Figure 2, the mobilities of the three deacylated derivatives became equal to that of the native enzyme. When the acylation was performed with 2.3 mol of H_4Pht anhydride/mol of amino groups, some dissociation of the protein occurred as revealed by the presence of two electrophoretic components. These results on the H_4Phtoyl derivatives of aldolase are very similar to those observed previously on the succinyl and citraconyl derivatives (Meighen and Schachman, 1970; Gibbons and Perham, 1970, 1974).

Reversible Acylation of Catalytic Subunit of ATCase. The addition of increasing amounts of H_4Pht anhydride to the native catalytic subunit (C_N) led progressively to inactivation of the protein with almost total loss of enzyme activity when there was an average of 5.5 H_4Phtoyl groups bound covalently per polypeptide chain (Table I). Derivative 4, containing an average of 4.6 H_4Phtoyl groups per catalytic chain, exhibited a single sharp symmetrical boundary in the ultracentrifuge with an $s_{20,w}$ of 5.1 S at a protein concentration of 5 mg/ml in 20 m*M* Tris-Cl–2 m*M* β-mercaptoethanol at pH 8.5. The H_4Phtoyl-catalytic subunit, C_T, recombined in high yield with an excess of regulatory subunit, R, to form ATCase-like molecules, $C_TC_TR_3$, having an $s_{20,w}$ of 10.2 S when dissolved at 5.1 mg/ml in the same buffer. Evidence demonstrating that there were two catalytic subunits in the reconstituted ATCase-like molecules was obtained from hybridization experiments (Meighen et al., 1970) which produced a three-membered, *inter*-subunit hybrid set when C_N and C_T were mixed with R. Figure 3 shows the electrophoretic patterns of C_N and the markedly altered derivative, C_T (number 5), along with the hybrid set formed in the reconstitution process. In this experiment the concentration of C_N was much larger than C_T; accordingly the resulting mixture contained more $C_NC_NR_3$ than $C_TC_TR_3$. Only a small amount of C_T is detectable in the pattern for the hybrid set; thus it appears that most of the acylated protein is competent for combination with R to form either $C_NC_TR_3$ or $C_TC_TR_3$.

Deacylation of C_T at 23° by incubation for 20 hr at 0.25 mg/ml in 50 m*M* phosphate buffer at pH 6 containing 2

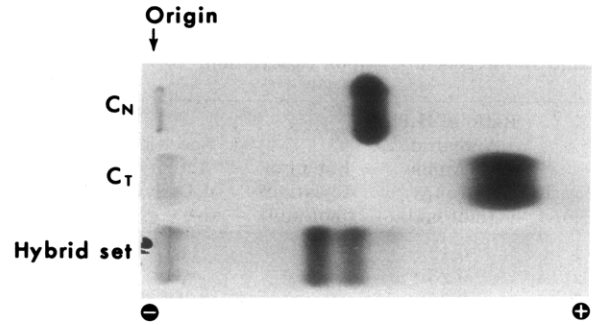

FIGURE 3: Electrophoresis patterns of native (C_N) and acylated (C_T) catalytic subunit of ATCase and *inter*-subunit hybrid set formed from C_N, C_T, and R. Patterns were obtained on cellulose acetate strips as described by Meighen et al. (1970). Hybridization was performed according to Meighen et al. (1970) by mixing 20 mg of C_N and 12 mg of C_T (derivative 5) and then adding 18 mg of R to give a total volume of 10 ml.

m*M* EDTA and 2 m*M* β-mercaptoethanol led to a product with an electrophoretic mobility on cellulose acetate strips indistinguishable from that of the native enzyme. Also as shown in Table I, 93% of the original enzymic activity was recovered for derivative 4 and 66% for number 5. Evidence for the removal of the H_4Phtoyl groups is shown by the electrophoresis patterns obtained with polyacrylamide gels containing 8 *M* urea at a pH about 9.5. The unmodified polypeptide chains (shown as A in Figure 4a) migrate as a major single band with a small amount of material having a slightly greater mobility.[3] Acylation of C_N produced the somewhat heterogeneous derivative (number 5) C_T, in which the polypeptide chains differed in the extent of modification. Evidence for this is presented in the electrophoresis pattern designated as B in Figure 4a. The H_4Phtoyl derivative in 8 *M* urea migrated as a series of discrete bands each corresponding to the modification of an integral number of amino groups. The spacing between the bands decreased slightly with increasing extent of acylation. Despite this effect there seems to be sufficient resolution of the bands to detect polypeptide chains differing from one another by only a single charge.

As seen in the electrophoresis pattern (C) for the deacylation derivative, the bulk of the material had a mobility identical with that of the unmodified protein (A). Only a small amount of material in the deacylated preparation had a mobility greater than that observed for the unmodified catalytic chains. It should be noted that these patterns were obtained with the most extensively acylated preparation (number 5 in Table I) and that the recovery of the enzymic activity after deacylation was only 66%. Thus some slight irreversible change may have occurred in this preparation in comparison to the less modified derivative (number 4) which upon deacylation showed almost a complete restoration of enzymic activity. The patterns for mixtures of unmodified and acylated protein (D) and of unmodified and deacylated protein (E) correspond to the sums of the respective individual patterns. Figure 4b shows a densitometer trace of the polyacrylamide–urea gel pattern (D) for the mixture of acylated and unmodified polypeptide chains.

[3] Calibration of the urea–polyacrylamide gels with succinylated catalytic subunit preparations indicates that this material has one additional negative charge compared to the bulk of the protein. This small amount of heterogeneity may be the result of deamidination of asparagine or glutamine residues of some of the polypeptide chains.

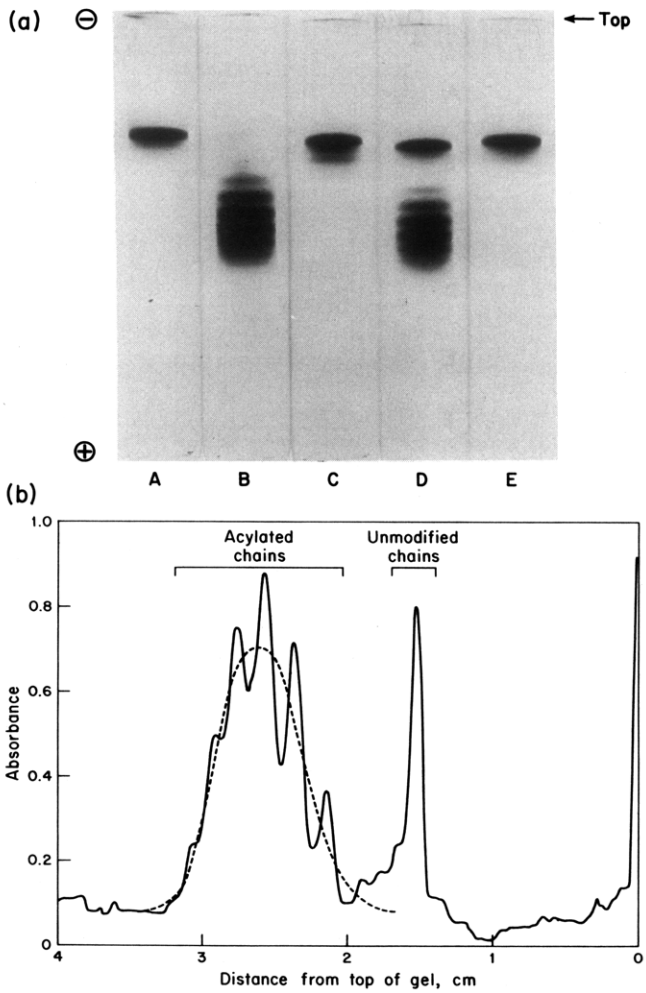

FIGURE 4: Electrophoresis patterns of polypeptide chains of catalytic subunit, the acylated derivative and the deacylated product. Electrophoresis in polyacrylamide gels containing 8 *M* urea was performed as described in Materials and Methods. The patterns in (a) are as follows: (A) unmodified catalytic subunit; (B) acylated catalytic subunit containing an average of 5.5 H_4Phtoyl groups per chain; (C) the deacylated product obtained by incubating the acylated derivative at 0.25 mg/ml for 20 hr at 23° in 50 m*M* phosphate buffer at pH 6 containing 2 m*M* EDTA and 2 m*M* β-mercaptoethanol; (D) a mixture of A and B; (E) a mixture of A and C. (b) Densitometer trace of the pattern in gel D. The dashed line is a Gaussian curve adjusted visually so as to approximate the distribution of acylated chains.

The predominant acylated species correspond to chains with 4, 5, and 6 H_4Phtoyl groups per polypeptide chain.

Use of H_4Phtoyl Groups as a Reversible "Chromatographic Handle". As a test of the H_4Phtoyl groups as a "chromatographic handle" we prepared a series of different ATCase-like hybrids which lost the characteristic allosteric properties of the native enzyme. These hybrids were then deacylated in order to determine whether the unusual kinetic and physical chemical behavior would be regained.

The *inter*-subunit hybrid, $C_NC_TR_3$, was isolated from the three-membered hybrid set (Figure 3) formed by mixing C_N and C_T with an excess of R. The fractionation by ion-exchange chromatography on a DEAE-Sephadex column (Meighen et al., 1970) is illustrated by the elution profile in Figure 5. The three members in the hybrid set are resolved along with the excess of R and the small amount of uncombined C_T. Electrophoresis on cellulose acetate strips was used to identify each of the components. After rechromatography of the fraction designated as $C_NC_TR_3$ this elec-

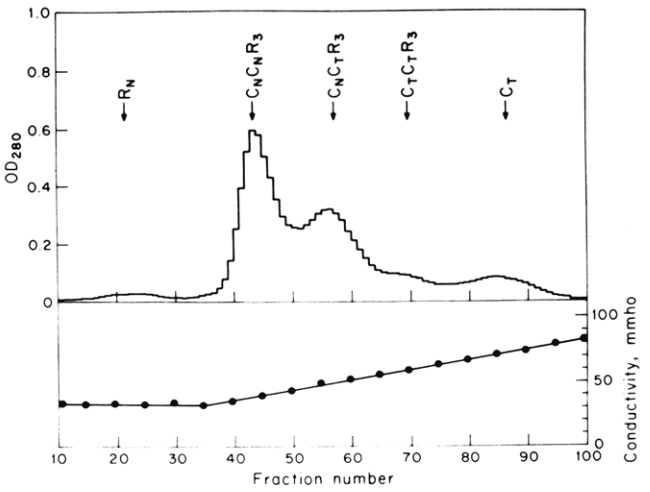

FIGURE 5: Chromatographic fractionation of hybrid set formed by reconstitution of ATCase-like molecules from C_N, C_T, and R. Chromatography on DEAE-Sephadex was performed as described by Meighen et al. (1970). The optical densities at 280 nm were measured for all fractions and are shown at the top of the diagram; the conductivity of the effluent is shown below. The hybrid set was that shown in Figure 3.

Table II: Kinetic Properties of *Inter*-Subunit Hybrid and Deacylated Product.

Species	V_{max}[a]	Hill Coefficient[a]	CTP Inhibition[b] (%)	K_m[c] (m*M*)
$C_NC_NR_3$[d]	8.3	1.6	56	6.5
$C_NC_TR_3$[e]	4.7	1.0	26	8.7
Deacylated $C_NC_TR_3$[f]	8.0	1.5	60	6.9
Native ATCase	11	1.8	70	7.0
Deacylated $C_TC_TR_3$[g]	11	1.8	62	7.0

[a] Values obtained from enzyme assays with [^{14}C]aspartate. Units of velocity are μmol of carbamyl aspartate per hr per μg of protein. [b] Inhibition produced by 0.5 m*M* CTP at an aspartate concentration of 5 m*M*. [c] K_m corresponds to the aspartate concentration for $V_{max}/2$. [d] Reconstituted ATCase formed in hybridization experiment and isolated by ion-exchange chromatography (see Figure 5). This product was exposed to same conditions used for deacylation of $C_NC_TR_3$. [e] Hybrid formed and isolated as described in Figure 3 and 5. [f] Deacylation performed as described in Table I. [g] Prepared by using C_T (sample 5 in Table I) for reconstitution with excess R followed by purification on DEAE-Sephadex and then deacylation as described in Table I followed by chromatography on Sephadex G-200.

trophoretic technique showed only a single species which had the kinetic properties summarized in Table II. In contrast to $C_NC_NR_3$ the hybrid, $C_NC_TR_3$, had a lower V_{max} due to a 50% reduction in the number of catalytically active chains, a Hill coefficient of 1.0 indicating no homotropic cooperativity, a significantly lower inhibition by CTP, and a slightly higher apparent K_m. After deacylation of the hybrid, $C_NC_TR_3$, at pH 6 (see Table I for experimental procedure) the product was examined by polyacrylamide gel electrophoresis. As seen in Figure 6, the electrophoretic pattern of the deacylated derivative (B) was virtually identical with that of $C_NC_NR_3$ (A). Moreover, as seen in Table II, the enzymic activity was restored as indicated by the increase of V_{max} from 4.7 to 8.0. In addition homotropic cooperativity was restored (Hill coefficient of 1.5) and the heterotropic effect manifested by CTP inhibition was increased to that of $C_NC_NR_3$. Finally, upon deacylation the apparent K_m decreased to a value similar to that of $C_NC_NR_3$.

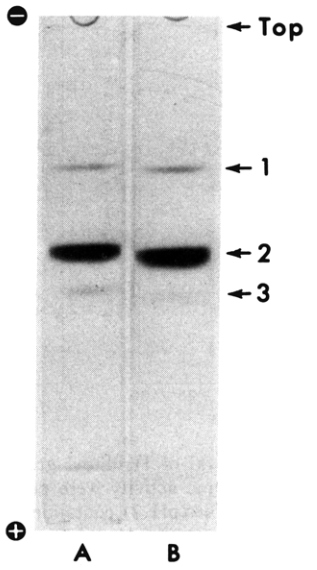

FIGURE 6: Electrophoresis pattern of reconstituted ATCase from the hybridization experiment and of deacylated $C_NC_TR_3$. Polyacrylamide gels were used for the electrophoresis as described in Materials and Methods. The pattern designated as A represents $C_NC_NR_3$ obtained from the chromatographic fractionation in Figure 5. The pattern labeled as B shows the deacylated product after treating $C_NC_TR_3$ by the procedure described in Figure 4. Positions labeled 1, 2, and 3 show the positions respectively of aggregated ATCase species (Gerhart and Schachman, 1968), ATCase, and regulatory subunit-deficient ATCase molecules (Yang et al., 1974).

Table II also presents data for the native enzyme and it is readily apparent in these experiments that the reconstituted ATCase-like molecules, $C_NC_NR_3$, differ somewhat from the native molecules in V_{max}, Hill coefficient, and apparent K_m. Since the $C_NC_NR_3$ molecules were formed in the same reconstitution experiment as the hybrid, $C_NC_TR_3$, the data for these two preparations were used for the above evaluation of the efficacy of the deacylation procedure. For reasons that are as yet not clairified, reconstituted ATCase-like molecules occasionally do not exhibit the same extent of homotropic and heterotropic effects as the native enzyme. Apparently some "damage" is incurred during the preparation of either or both of the individual subunits. Hence an additional experiment was performed to determine whether the properties of the native enzyme could be regenerated from inactive H_4Phtoyl-catalytic subunits. The virtually inactive derivative, C_T (number 5 in Table I), was mixed with R and the resulting mixture was chromatographed on DEAE-Sephadex to give $C_TC_TR_3$ containing a small amount of free C_T. Deacylation was then performed to produce $C_NC_NR_3$ and some C_N which were separated by Sephadex G-200 chromatography. The deacylated ATCase-like product, as seen in the last row of Table II, had kinetic properties almost identical with those of the native enzyme. Not only was full activity regenerated from inactive material but also the cooperativity was completely restored.

The effectiveness of the deacylation procedure was tested with another *inter*-subunit hybrid containing one fully active, native catalytic subunit, C_N, and one subunit which was specifically inactivated by reaction with pyridoxyl 5′-phosphate followed by reduction of the Schiff base with $NaBH_4$ (Greenwell et al., 1973). Since the charge on the pyridoxylated subunit, C_P, is not sufficiently different from that of C_N, the members of the hybrid set formed from C_N,

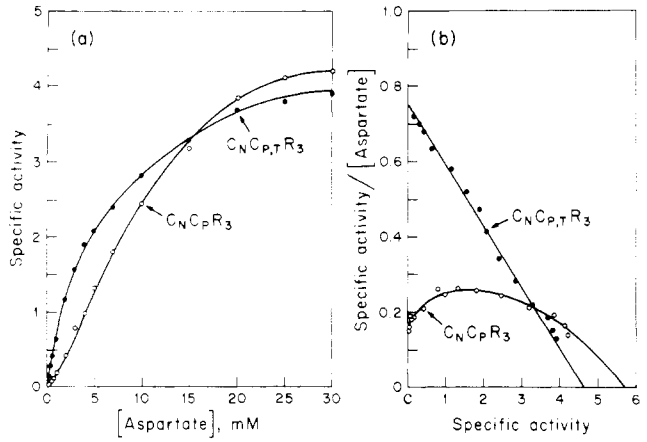

FIGURE 7: Effect of removal of H_4Phtoyl groups from the hybrid, $C_NC_{P,T}R_3$. Assays of enzymic activity were performed at 30° in 50 m*M* imidazole acetate buffer (pH 7) containing 0.2 *M* EDTA. Carbamyl phosphate was present at 4 m*M* and the concentration of aspartate was varied. The hybrid, $C_NC_{P,T}R_3$, was prepared according to Gibbons et al. (1974) and deacylation of it to give $C_NC_PR_3$ was performed as described in Table I. The specific activity is given as μmol of carbamyl aspartate formed per hr per μg of protein. (a) The saturation curve of specific activity vs. aspartate concentration. (b) The data plotted as specific activity/aspartate concentration vs. specific activity (Eadie, 1942). Values for $C_NC_{P,T}R_3$ designated by ● and for $C_NC_PR_3$ by ○.

C_P, and R could not be resolved. Accordingly Gibbons et al. (1974) modified C_P with H_4Pht anhydride to give $C_{P,T}$ which was then used in the hybridization experiment with C_N and R so as to permit the isolation of $C_NC_{P,T}R_3$. As seen in Figure 7a, which presents kinetic data at different substrate concentrations, the hybrid, $C_NC_{P,T}R_3$, exhibits a hyperbolic dependence of its activity on aspartate concentration. This is shown clearly by the straight line in Figure 7b when the specific activity divided by aspartate concentration is plotted against the specific activity (Eadie, 1942). Deacylation of that derivative yielded $C_NC_PR_3$ which exhibited cooperativity as illustrated by the sigmoidal dependence of enzyme activity on aspartate concentration and the marked curvature (and maximum) in the Eadie plot (Figure 7a and b).

The efficacy of the deacylation procedure was also tested with the *intra*-subunit hybrid, $C_{nnt}C_{nnt}R_3$. This hybrid and the deacylated product were used for sedimentation studies to determine whether the ligand-promoted conformational changes were similar to those characteristic of the native enzyme (Gerhart and Schachman, 1968). As seen in Table III the value of $\Delta s/\bar{s}$ upon the addition of the substrate, carbamyl phosphate, to $C_{nnt}C_{nnt}R_3$ was −2.0%, a value much larger than that observed with the native enzyme. Conversely the change accompanying the subsequent addition of the competitive inhibitor, succinate, was much smaller for the hybrid than for ATCase. Upon deacylation the product exhibited the same values of $\Delta s/\bar{s}$ as native ATCase when the various ligands were added.

Table III: Effect of Ligands on Sedimentation Coefficient of ATCase and Acylated and Deacylated Derivatives.

Species	$\Delta s/\bar{s}$ in %[a]		
	Carbamyl Phosphate[b]	Carbamyl Phosphate + Succinate[c]	Succinate[d]
ATCase	−0.5	−3.6	−3.1
$C_{nnt}C_{nnt}R_3$	−2.0	−2.4	−0.4
Deacylated $C_{nnt}C_{nnt}R_3$[e]	−0.2	−3.3	−3.1

[a] Measured according to Gerhart and Schachman (1968). The solvent was 40 m*M* phosphate buffer at pH 7 containing 0.2 m*M* EDTA and 2 m*M* β-mercaptoethanol. [b] Ligand concentration was 2 m*M*. The reference solution contained 2 m*M* additional phosphate to compensate for the viscosity and density contributed by the ligand. [c] Ligand concentrations were 2 m*M*. The reference solution contained 2 m*M* additional phosphate and 2 m*M* glutarate to compensate for the carbamyl phosphate and succinate. [d] Data obtained by subtraction of those in the other two columns. They represent the effect of succinate in the presence of carbamyl phosphate. [e] Deacylation was performed as described in Table I.

Discussion

Meighen and Schachman (1970) have discussed criteria which must be satisfied if a chemically modified variant is to be suitable for hybridization with an unmodified oligomeric species. These may be summarized as follows. (1) The modified protein should be reasonably homogeneous. (2) It should be readily separable from the native protein by available fractionation procedures. (3) The modification should result in a product with tertiary and quaternary structures similar to those of the native oligomer and the interaction energies among the subunits should not be drastically altered. (4) The subunits produced upon dissociation of the modified oligomer should be able to recombine equally well with each other *and* with the native subunits to produce oligomeric structures similar to the native enzyme. A corollary of the last requirement is that the isolated hybrids should not disproportionate to give other hybrids or parental species.

In addition to these requirements, a reversible "chromatographic handle" must satisfy three other criteria. First, the modifying groups must remain attached to the protein for the time needed to complete the preparation and fractionation of the hybrid set. Since the formation of the hybrid set frequently requires the dissociation of the oligomeric proteins and then reconstitution, the new covalent linkage must be stable in concentrated urea or guanidine hydrochloride solutions. Second, the removal of the groups must be accomplished under conditions which do not cause the dissociation of the oligomers into subunits or permit the disproportionation of the hybrids. Since many oligomeric proteins are dissociated in mildly acid or alkaline solutions, the pH of the solutions must be maintained near neutrality. Third, the modification procedure must be sufficiently specific that no side reactions occur which preclude the restoration of biological function after removal of the groups. As shown in the numerous tests presented above for aldolase and the catalytic subunit of ATCase, modification with H_4Pht anhydride fulfills all these criteria and the H_4Phtoyl groups serve as an effective and efficient reversible "chromatographic handle" for the preparation of hybrids of unusual composition and structure.

As shown by Kirby and Lancaster (1972) the rates of deacylation of substituted maleamic acids vary considerably depending on the number, size, and type of substituents at the carbon–carbon double bond. Both maleic anhydride and 2-methylmaleic (citraconic) anhydride are not satisfactory reversible acylating reagents for the production of protein hybrids because the deacylation rates at pH values near neutrality are much too slow. Hence the effective removal of these acyl groups would require lowering the pH to values which would cause the dissociation of many oligom-

eric proteins. Conversely the protein derivative formed with 2,3-dimethylmaleic anhydride deacylates so rapidly at neutral pH as to preclude the hybridization and chromatographic fractionation required to produce the desired species. When, however, the two alkyl groups on the carbon–carbon double bond are joined in a ring as in H_4Pht anhydride, the deacylation rate of the acylated derivative is reduced considerably (Kirby and Lancaster, 1972). Generally fractionation procedures designed to resolve hybrids of oligomeric proteins employ buffers of pH 7–9 and require of the order of 1 day for completion; thus deacylation rates with half-times of 2 days in the region of pH 7 would be appropriate. The H_4Phtoyl derivatives of aldolase and the catalytic subunit of ATCase seem to have both the requisite stability at the higher pH and a satisfactory deacylation rate at pH 6.

The extent of reaction of the amino groups in C_T (derivative 5) was calculated from the spectrum (Figure 1) and the value, 5.4 H_4Phtoyl groups per chain, was in excellent agreement with the number of blocked amino groups measured by the TNBS assay (5.5 groups per chain). A similar result was deduced from the electrophoresis experiments on polyacrylamide gels containing 8 *M* urea. The agreement among these three different determinations provides evidence that acylation of amino groups was the predominant reaction and that there was little or no esterification of seryl or threonyl side chains. Moreover, treatment of C_T with 0.5 *M* hydroxylamine in borate buffer at pH 9, which would have led to the decomposition of O esters and a consequent change in electrophoretic mobility, had no effect on the electrophoresis pattern. Apparently H_4Pht anhydride did not react with the sulfhydryl groups[4] in C_T since the sulfhydryl content of the derivative was 0.99 group per polypeptide chain in agreement with the cysteinyl composition of the native subunit (Vanaman and Stark, 1970).

Although quantitative comparisons were not made, it appears that less H_4Pht anhydride is required to produce the same extent of acylation of proteins than would be needed with succinic, maleic, or citraconic anhydrides. This efficiency as an acylating agent may be related to the greater hydrophobic character of H_4Pht anhydride which has three more CH_2 groups than citraconic anhydride and hence reacts preferentially with protein nucleophiles rather than with water or hydroxyl ions. Although H_4Pht anhydride reacted only with the amino groups of the catalytic subunit of ATCase, the resulting derivative did not recombine completely with regulatory subunits to give ATCase-like molecules. This decrease in reconstitutability may be attributable to steric effects resulting from the bulky H_4Phtoyl groups on the protein.

The heterogeneity of C_T was clearly demonstrated by electrophoresis in the polyacrylamide–urea gels where the tertiary and quaternary structures were destroyed and the mobility was a function of the net charge on the individual polypeptide chains. As seen in Figure 4, for C_T containing an average of 5.5 H_4Phtoyl groups per chain there were chains containing as few as three and as many as eight acyl groups per chain. Most of the polypeptide chains contained 4, 5, or 6 H_4Phtoyl groups per chain. When electrophoresis of the acylated intact catalytic trimers (Figure 3) was conducted on cellulose acetate strips the apparent heterogeneity was much less. This apparent difference in heterogeneity is due in part to the characteristics of the staining procedure which in gels tends to emphasize the minor (outer) bands (see Figure 4b) and in strips disguises the extent of spreading of the bands. The regular spacing of the individual acylated catalytic chains in the polyacrylamide–urea gels indicates that this electrophoretic technique, suitably calibrated with molecules of known charge, might be useful in estimating the net charge of polypeptide chains. A plot of the mobilities of the bands vs. the numbers of acyl groups on the chains indicated that the mobility increased by about 7% per unit change in the net charge. This same value was obtained (K. Wall, unpublished observations) in studies of the catalytic chains alkylated at the single cysteinyl residue with iodoacetate and iodoacetamide.

Both the number and intensity of the bands in the polyacrylamide–urea gels and the simulated Gaussian curve (Figure 4b) can be used to estimate the heterogeneity of C_T. Although quantitative determinations would be hazardous, it appears that the inhomogeneity produced by acylation was significantly less than that expected if all the amino groups reacted equally rapidly with H_4Pht anhydride. The observed width of the Gaussian curve was about 60% of that estimated for a random reaction with any of the amino groups in the polypeptide chains. Such a result would be obtained if the amino groups existed in "subclasses" of differing reactivities (Meighen and Schachman, 1970). The observed heterogeneity of C_T could be accounted for by assuming that there are seven highly reactive and eight less reactive amino groups per polypeptide chain. Similarly the inhomogeneity can be estimated from the widths of the chromatographic peaks of the acylated derivatives as compared to the unmodified species if it is assumed that all the additional charged groups contribute equally to the binding of the hybrids to the ion-exchange media. Although this assumption may not be valid in a quantitative sense it is interesting to note that the individual species in the hybrid sets of ATCase were almost evenly spaced and that the *inter*-subunit hybrid, $C_NC_TR_3$, and the *intra*-subunit hybrid, C_{nnt}, were resolved readily by ion-exchange chromatography (e.g., Figure 2 in Gibbons et al., 1974). Heterogeneity produced by acylation becomes detrimental to the chromatographic fractionation when the spread of charges in one hybrid approaches the average charge difference between adjacent members of the hybrid set. With some proteins the acylated derivatives may be so heterogeneous that a preliminary fractionation of the parental, modified species may be required (Gibbons, 1974).

All of the acylated hybrids, $C_NC_TR_3$, $C_NC_{P,T}R_3$, and $C_{nnt}C_{nnt}R_3$, lack the cooperativity characteristic of the native enzyme. Similarly ATCase-like molecules containing two native catalytic subunits and three extensively succinylated regulatory subunits (Nagel and Schachman, 1975) are devoid of cooperativity. Presumably this loss of allosteric properties can be attributed to the repulsive interactions caused by the negatively charged acylated side chains in the protein. Since the energy difference between the putative constrained (low affinity) and relaxed (high affinity) states of the native enzyme is likely to be low (Howlett, G. J., Blackburn, M. N., and Schachman, H. K., unpublished observations) the additional electrostatic repulsion from the acyl groups may destabilize the constrained form of the enzyme relative to the relaxed state. As a consequence the molecules would undergo a conformational change related to the allosteric transition either spontaneously or upon the binding of carbamyl phosphate. Also the subsequent addi-

[4] Acylation of sulfhydryl groups by H_4Pht anhydride probably does occur followed by rapid deacylation.

tion of the substrate, aspartate, or the analogue, succinate, would have little effect since the transition of the enzyme molecules to the relaxed state would have already occurred. The measurements of $\Delta s/\bar{s}$ (Table III) for $C_{nnt}C_{nnt}R_3$ showed that the ligand-promoted conformational change with carbamyl phosphate was much larger than that with the native enzyme; conversely a very small change occurred upon the subsequent addition of succinate to the hybrid. Similar results were obtained with $C_NC_{P,T}R_3$ as compared to $C_NC_PR_3$ (Gibbons et al., 1974). Upon deacylation of these derivatives (Table III, and Table I in Gibbons et al., 1974) the pattern of ligand-promoted conformational changes was similar to that exhibited by the native enzyme. Thus removal of the acyl groups from the acylated AT-Case-like molecules led to the restoration not only of the allosteric kinetic behavior but also of the ligand-promoted conformational changes characteristic of the native enzyme.

Depending upon the stability of the protein and the time and conditions required to complete the hybridization and fractionation it may prove desirable to use anhydrides with properties different from H_4Pht anhydride. As yet only a few of the "reversible" anhydrides have been exploited in protein chemistry and others (Kirby and Lancaster, 1972) may well be superior for a specific experimental application.

Acknowledgments

We thank J. E. Flatgaard and A. J. Kirby for invaluable suggestions and Y. R. Yang and B. Yee for skillful help in conducting some of the experiments.

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