Conformational Studies on the Nitrated Catalytic Subunit of Aspartate Transcarbamylase†

Marc W. Kirschner‡ and H. K. Schachman*

ABSTRACT: A variety of physical–chemical techniques such as difference sedimentation, difference spectroscopy, and optical rotatory dispersion have shown that the isolated catalytic subunits of aspartate transcarbamylase undergo changes in conformation upon the addition of specific ligands. However these methods were not sufficiently discriminating to provide definitive evidence for the existence of comparable (or different) ligand-promoted conformational changes in these subunits when they are incorporated within intact enzyme molecules. Hence efforts were directed toward attaching a specific, sensitive chromophore onto the catalytic polypeptide chains so that a selective spectral probe would be available for such investigations. Accordingly studies were conducted on the nitration of the isolated catalytic subunits with tetranitromethane. Under conditions which minimized side reactions a derivative was obtained having 0.7–0.9 nitrotyrosyl residue/polypeptide chain. The nitrated catalytic subunits had enzymic properties similar to those of the native subunit; moreover, the modified subunits combined in high yield with native regulatory subunits to give nitrated aspartate transcarbamylase. Upon the addition of either the substrate, carbamyl phosphate, or the inhibitor, succinate, there was no change in the spectrum of the nitrated catalytic subunit. In the presence of both ligands, however, the spectrum of the chromophore was altered markedly with a 14% decrease in absorbance at 430 nm. Studies with other ligands showed that spectral changes occurred only with those ligand pairs which caused conformational changes as measured by other methods. Titration of the spectral change as a function of succinate concentration in the presence of carbamyl phosphate yielded a hyperbolic curve with a dissociation constant of 3.7×10^{-4} M. This value is similar to those obtained from direct binding experiments, from enzyme kinetics, and from measurements of conformational changes of the native subunit. These results indicate that conformational changes in the isolated catalytic subunits are closely linked to the binding of ligands.

Both aspartate transcarbamylase and the free catalytic subunits isolated from it undergo conformational changes upon the addition of substrates and substrate analogs (Gerhart and Schachman, 1968; McClintock and Markus, 1968, 1969; Collins and Stark, 1969; Kirschner and Schachman, 1971b). However, it is not known whether, and to what extent, the catalytic subunits within the intact enzyme molecules change upon the addition of ligands. On the one hand, we might visualize a model in which the local changes in the catalytic subunits within the intact enzyme are similar to those observed in the isolated subunits and are linked to gross and different transitions in the enzyme complex. Such a model could account for the hydrodynamic studies (Kirschner and Schachman, 1971b) which showed that the ligand-promoted change in the sedimentation coefficient of the isolated catalytic subunits was opposite in direction (and smaller in magnitude) to that of the intact enzyme. On the other hand, we might consider a model in which the quaternary structure of aspartate transcarbamylase constrains the catalytic subunits into a conformation different from that of the isolated subunits. For this model the conformational transition of the intact enzyme could represent merely the sum of the changes experienced by the individual subunits which differ from those observed with the isolated subunits. In attempting to distinguish between these alternative models we have employed several physical–chemical techniques to determine the effects of ligands on the isolated catalytic subunits and compare them to the effects on the native enzyme.

A combination of techniques is required since the various methods may reflect *different* effects, such as changes in the catalytic subunit near or distal to the ligand binding site or changes in the packing of the subunits in the enzyme complex. Hydrodynamic methods, though free of some of the ambiguities of spectral methods, cannot distinguish readily conformational changes in one subunit independent of another. Spectral methods can also be ambiguous if, in different subunits of the molecule, there are a number of chromophores with overlapping spectra. In aspartate transcarbamylase, the tryptophanyl residues would constitute an ideal spectral probe since they are present only in the catalytic subunits (Weber, 1968a,b; Changeux and Gerhart, 1968). However, there have been no unequivocal studies showing changes in their absorption or fluorescence upon the addition of specific ligands. Hence we turned to the tyrosyl residues which were shown by Collins and Stark (1969) to have altered absorption properties when ligands were added to the isolated catalytic subunits. In order to differentiate among the tyrosyl residues in the two types of subunits, we nitrated some of the tyrosyl residues in the isolated catalytic subunits and then used these nitrated catalytic subunits with native regulatory subunits to form reconstituted enzyme-like molecules.

Nitration of tyrosyl residues with tetranitromethane has been shown to be a mild and relatively specific method for modifying proteins (Sokolovsky *et al.*, 1966; Riordan *et al.*, 1967; Vallee and Riordan, 1969). Since the spectrum of the

† From the Department of Molecular Biology and the Virus Laboratory, Wendell M. Stanley Hall, University of California, Berkeley, California 94720. *Received October 25, 1972.* This research was supported by Public Health Service Research Grant GM 12159 from the National Institute of General Medical Sciences and by National Science Foundation Research Grant GB 4810X.

‡ Predoctoral Fellow of the National Science Foundation (1968–1971). This material was submitted in partial fulfillment of the Ph.D. requirements in Biochemistry at the University of California, Berkeley, California. Present address: Department of Biochemical Sciences, Princeton University, Princeton, N. J. 08540.

nitrotyrosyl residue changes markedly from the ionized to the un-ionized form, this chromophore should be a sensitive probe for detecting small changes in its local environment at neutral pH. Indeed Riordan *et al.* (1967) and Cuatrecasas *et al.* (1968), in studies of the nitrated derivatives of carboxypeptidase A and staphylococcal nuclease, repectively, found changes in the nitrotyrosyl spectrum due to binding of specific ligands. For the studies presented in this and the following paper the nitrated catalytic subunit of aspartate transcarbamylase must have certain properties. First, the absorption spectrum of the nitrotyrosyl groups should be sensitive to the binding of substrates and their analogs. Second, the nitrated derivative should have enzymic and physical properties similar to those of the native protein. Third, the nitrated derivative should be homogeneous so that spectral changes could be attributed to chromophores occupying specific positions in all molecules. Fourth, the nitrated derivative of the catalytic subunit should be capable of combining with native regulatory subunits to form enzyme-like complexes.

We have found, as have others, that care must be taken to avoid complicating side reactions when proteins are treated with tetranitromethane. However, under certain conditions it has been possible to nitrate nearly one tyrosyl residue per chain of the catalytic subunits with only a small loss of enzymic activity. This nitrated derivative of the catalytic subunit has an absorption spectrum which is altered markedly upon the addition of specific ligands. Also the nitrated catalytic subunits when added to native regulatory subunits form complexes which are similar in enzymic behavior and physical properties to native aspartate transcarbamylase. This paper describes the production and behavior of the nitrated catalytic subunit. In the following paper (Kirschner and Schachman, 1973) we present results of studies on the reconstituted nitrated enzyme complex showing that the nitrotyrosyl chromophore serves as a useful probe for detecting local conformational changes in the enzyme.

Experimental Section

Materials. Cytidine triphosphate, D-aspartic acid, L-aspartic acid, malonic acid, D-malic acid, L-malic acid, and glutaric acid purchased from Sigma Chemical Co. and succinic acid purchased from Eastman Organic Chemicals were used without further purification. Bovine serum albumin was obtained from Armour (lot D71002). The dilithium salt of carbamyl phosphate, obtained from Sigma Chemical Co., was reprecipitated from cold ethanol and water (Gerhart and Pardee, 1962). Owing to its instability in aqueous solutions, fresh solutions were prepared before each experiment. The mercurials, *p*-hydroxymercuribenzoate, obtained from Sigma Chemical Co. and 1-(3-chloromercuri)-2-methoxypropyl)urea, (neohydrin), obtained from K & K Laboratories, were each purified by reprecipitation from alkaline solutions by the addition of hydrochloric acid. Nitrotyrosine, obtained from K & K Laboratories, was recrystallized three times from water and dried over anhydrous $CaCl_2$. It had an extinction coefficient at 430 nm of 4150 l. mol^{-1} cm^{-1} at pH 8 and gave a color yield by amino acid analysis of 94% that for isoleucine (average of five determinations). Tetranitromethine was obtained from Aldrich Chemical Co. It contained some nitroformate ion impurity as judged by its yellow color. The nitroformate ion and possibly other water-soluble impurities could be removed by extraction of tetranitromethane with equal volumes of water ten times. The purified material showed no difference from the unpurified sample in its rate of reaction or in the characteristics of the products, so that in later experiments tetranitromethane was used without this treatment. DEAE-cellulose was obtained from Eastman Organic Chemicals and washed according to the procedure of Peterson and Sober (1962). Phosphonacetamide was a generous gift of Gregg Davies and George Stark.

Nitration of the Catalytic Subunit. In the procedure adopted for the nitration of the catalytic subunit, the protein was dialyzed *vs.* 0.1 M potassium phosphate buffer at pH 6.7 containing 0.5 M KCl and 2 mM EDTA. The protein was diluted to a final concentration of 2–4 mg/ml and succinate and carbamyl phosphate, in the same buffer and at the same pH, were added to a final concentration of 0.05 and 0.02 M, respectively. To 1 ml of the protein solution 20 μl of 0.5 M tetranitromethane in 95% ethanol was added with constant stirring. After 75 min at room temperature, the reaction was quenched by the addition of 50 μl of 1 M mercaptoethanol. The protein was immediately dialyzed against two changes of 500 ml of 0.04 M potassium phosphate buffer at pH 7 containing 2 mM mercaptoethanol and 0.2 mM EDTA. In other experiments the amount of tetranitromethane, the nature of the ligands, the pH, buffer, and time of reaction were varied.

Spectral Determination of Sulfhydryl Groups and Nitrotyrosyl Groups. Protein concentrations were determined in either of two ways. One involved measuring the refractive index increment in a synthetic boundary experiment in the ultracentrifuge, assuming that a displacement of four fringes corresponded to a concentration of 1 mg/ml for a cell with a 12-mm optical path length (Richards and Schachman, 1959; Babul and Stellwagen, 1969). Alternatively protein concentrations were determined by the method of Lowry *et al.* (1951) using crystalline bovine serum albumin as a standard. Sulfhydryl determinations were performed by the method of Sedlak and Lindsay (1968) using Nbs_2[1] (Ellman, 1959). For spectral determination of the number of nitrotyrosyl groups an aliquot of the nitrated catalytic subunit (0.5 ml) in 0.1 M Tris-HCl buffer at pH 8.2 containing 2 mM EDTA was added to an equal volume of buffer in 8 M guanidine hydrochloride. The concentration of nitrotyrosyl groups was calculated from the absorbance at 428 nm using an extinction coefficient of 4.1×10^3 M^{-1} cm^{-1} (Riordan *et al.*, 1967).

Amino Acid Analyses. Amino acid analyses were performed with a Spinco Model 120B automatic amino acid analyzer. Aliquots of protein were dissolved in 2 ml of 6 M HCl containing 2 mg of phenol to increase the yields of tyrosine and nitrotyrosine (Boesel and Carpenter, 1970). The samples were hydrolyzed in evacuated tubes at 110° for 20 hr. In this period of time there was a 3% loss of tyrosine and a 5% loss of nitrotyrosine. All values for the amount of tyrosine and nitrotyrosine were corrected for this loss.

Miscellaneous Methods. Difference spectra were obtained with a Cary 14 spectrophotometer equipped with a high-intensity tungsten source, a microcell attachment and a 0–0.1 absorbance expanded-scale slide-wire. The reference attenuator was modified to accommodate a microcell holding small volumes (0.1 ml). In difference spectral titrations aliquots of ligand solutions were added to the microcell in the sample compartment and identical volumes of buffer were added to the microcell in the reference compartment using a Hamilton syringe fitted with a Cheney adapter (The Hamilton Co., Inc., Whittier, Calif.). The spectra were measured at temperatures of 20–22°.

[1] Abbreviation used is: Nbs_2, 5,5′-dithiobis(2-nitrobenzoic acid).

TABLE I: Protection of Sulfhydryl Groups on the Catalytic Subunit.[a]

Expt	TNM[b] (mM)	PMB[c] (mM)	Carbamyl Phosphate (mM)	Act. (%)	SH Groups (No./Subunit)	Nitrotyrosine (No./Subunit)
1	0	0	0	100	3.2	0
2	0	0.2	0	87	2.9	0
3	0	0.2[d]	0	97	3.2	0
4	0	2	0	70	2.4	0
5	2	2	0	9	2.1	6.9
6	2	0	4	18	2.9	3.9
7	2	0	0	<0.1	0	7.2

[a] Catalytic subunit at a concentration of 3 mg/ml in 0.1 M Tris-acetate at pH 8 containing 0.2 mM EDTA was incubated for 2 hr with the reagents indicated. Enzymic activity was measured at pH 7 in solutions containing 0.02 M aspartate and 4 mM carbamyl phosphate; spectral determinations of the number of nitrotyrosyl groups, and titration of the number of sulfhydryl groups were performed as described in Methods. [b] TNM = tetranitromethane. [c] PMB = *p*-hydroxymercuribenzoate. [d] For this experiment 0.2 mM neohydrin was substituted for *p*-hydroxymercuribenzoate. High concentrations of neohydrin (2 mM) caused the protein to precipitate after addition of mercaptoethanol.

All ultracentrifuge experiments were conducted with a Beckman-Spinco Model E ultracentrifuge equipped with a schlieren and Rayleigh interference optical system (Svensson, 1950; Richards and Schachman, 1959). Difference sedimentation experiments were conducted according to the method of Richards and Schachman (1959) and Kirschner and Schachman (1971a). All experiments were at 20°.

Polyacrylamide gel electrophoresis was performed according to the method of Weber and Osborn (1969) and Shapiro *et al.* (1967).

Results

Reaction of Tetranitromethane with the Sulfhydryl and Tyrosyl Groups on the Catalytic Subunit. Vanaman and Stark (1970) in an exhaustive study of the sulfhydryl groups on the catalytic subunit showed that there were three per subunit or one per polypeptide chain. They also found that these sulfhydryl groups were quite unreactive, being essentially inert to alkylating agents and reacting only slowly with Nbs_2. From a variety of studies they concluded that the sulfhydryl groups, while not required for activity, are probably located near the active site. Since tetranitromethane is known to react readily with sulfhydryl groups as well as with tyrosyl residues (Sokolovsky *et al.*, 1966; Riordan and Christen, 1968; Sokolovsky *et al.*, 1969), it was important to determine whether, during the course of the reaction with tyrosyl residues, the sulfhydryl groups were also affected and, if necessary, to find ways to protect them during the reaction.

Addition of tetranitromethane to catalytic subunit at pH 8 led to a rapid loss of sulfhydryl groups as judged by subsequent titration of the protein with Nbs_2 under dissociating conditions. There was a slower production of nitrotyrosyl groups as judged spectrally. The product obtained 10 min after the addition of tetranitromethane had 2 nitrotyrosines/subunit, less than 0.2 sulfhydryl group/subunit, and less than 5% of its original enzymic activity. The total number of nitrotyrosyl groups after 2 hr was 6.8/subunit or approximately 2.3/chain. The resulting protein was totally inactive and would not recombine with regulatory subunit under the usual conditions as judged by electrophoresis on cellulose acetate (Gerhart and Schachman, 1956; Meighen *et al.*, 1970).

The sulfhydryl groups on the catalytic subunit may be protected from reaction with tetranitromethane by first blocking them with *p*-hydroxymercuribenzoate. As shown in Table I (expt 2 and 4), incubation of the catalytic subunit with *p*-hydroxymercuribenzoate (at 2- and 20-fold molar excess) led to a small, irreversible loss of sulfhydryl groups as judged by subsequent titration with Nbs_2. When carbamyl phosphate was present in the solution during the nitration in place of *p*-hydroxymercuribenzoate, there was very little loss of sulfhydryl groups as seen in expt 6 of Table I. Moreover, the presence of carbamyl phosphate decreased the total amount of nitrotyrosine produced in 2 hr of reaction (compare expt 5 and 6). For all subsequent nitration studies carbamyl phosphate was used instead of *p*-hydroxymercuribenzoate to protect the sulfhydryl groups.

Cross-Linking of Catalytic Subunits upon Reaction with Tetranitromethane. The protein obtained by nitration at pH 8

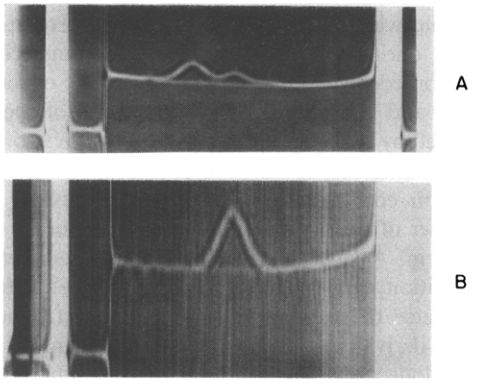

FIGURE 1: Sedimentation velocity patterns of nitrated catalytic subunit. The top photograph shows a schlieren pattern for nitrated catalytic subunit prepared by reacting catalytic subunit with 2×10^{-3} M tetranitromethane in 0.1 M Tris-acetate buffer at pH 8 containing 4.0×10^{-3} M carbamyl phosphate for 1 hr. The protein concentration was 4 mg/ml and the angle of the schlieren diaphragm was 70°. The bottom photograph shows a similar pattern for nitrated catalytic subunit prepared at pH 7 in 0.1 M imidazole acetate buffer after reaction with 0.01 M tetranitromethane. The protein concentration was 2.8 mg/ml and the angle of the schlieren diaphragm was 60°. Both samples were in 0.04 M potassium phosphate (pH 7), containing 2×10^{-3} M mercaptoethanol and 2×10^{-4} M EDTA. The rotor speed was 60,000 rpm and the temperature 20°.

TABLE II: Effect of pH on Reaction of Tetranitromethane with Catalytic Subunit.[a]

pH	Rate[b]	Act.[c]	Nitrotyrosine[d] (No./Subunit)	Amount of Dimer[e] (%)
9.0	1.00	5	5.4	44
8.5	0.64	10	4.5	42
8.0	0.36	22	4.2	36
7.5	0.17	31	3.6	5
7.0	0.05	65	2.4	0
6.7	0.036	72	1.5	0
6.7[f]	0.021	90	0.9	0

[a] The nitration reaction was carried out at a protein concentration of 3 mg/ml with 5×10^{-3} M tetranitromethane in buffered solutions containing 0.2 M KCl, 0.2 mM EDTA, and 0.004 M carbamyl phosphate for 1 hr. For pH 9, 8.5, and 8, 0.1 M Tris-acetate buffer was used. For pH 7.5, 7, and 6.7, 0.1 M imidazole acetate was employed. [b] The initial rate of the reaction is expressed relative to that at pH 9. The rate was measured at different intervals of time spectrally by the appearance of trinitroformate ion absorbance at 350 nm as described by Sokolovsky *et al.* (1966). [c] Enzymic activity remaining after 1 hr of reaction was measured at a concentration of 0.01 M aspartate. It is expressed as a percentage of that for the unreacted protein. [d] The amount of nitrotyrosine per subunit, produced after 1 hr of reaction, was determined spectrally. [e] The amount of dimer was determined by measurement of the areas corresponding to the boundaries in a sedimentation velocity experiment at 60,000 rpm with schlieren optics. The amount of dimer is expressed as a percentage of the total amount of protein. [f] Along with all the other compounds succinate was present at a concentration of 0.02 M during the nitration.

in the presence of carbamyl phosphate was inhomogeneous as seen by the sedimentation velocity pattern in Figure 1A. Similar results were obtained when *p*-hydroxymercuribenzoate was present during the nitration. The slower material had a sedimentation coefficient of 5.7 S, characteristic of the catalytic subunit, and it accounted for about 60% of the protein as determined by the areas corresponding to the two boundaries after correction for radial dilution. The faster species with a sedimentation coefficient of 8.9 S was consistent with its being a dimer of the slower material and accounted for 35% of the protein. A small amount of material sedimented still faster. Addition of high concentrations of mercaptoethanol did not reverse the formation of dimers. Electrophoresis of the nitrated derivative on polyacrylamide gels in the presence of sodium dodecyl sulfate produced three major bands with mobilities corresponding to molecular weights of 3.5×10^4, 7.4×10^4, and 8.0×10^4. A minor band corresponded to molecular weight of 1.1×10^5. The two bands with apparent molecular weights of 7.4×10^4 and 8.0×10^4 may represent cross-linked dimers with slightly different mobilities. The same bands were present when, prior to its application to the gel, the nitrated protein was incubated in 8 M urea with sodium borohydride or mercaptoethanol at a temperature of 50° for 2 hr. The 8.9S species thus seems to be a covalently cross-linked dimer produced by the action of tetranitromethane; disulfide bonds do not appear to be implicated. A dimer of mol wt 7×10^4 would be produced by one cross-link between two chains on each subunit. The production of such dimers by the action of tetranitromethane has been demonstrated previously in immunoglobins by Doyle *et al.* (1968), in insulin by Boesel and Carpenter (1970), and in a variety of proteins by Vincent *et al.* (1970). The extent of cross-linking was strongly dependent on the pH at which the reaction was conducted as demonstrated below.

Effect of pH on Reaction with Tetranitromethane. As shown in Table II, the rate of reaction of catalytic subunit with tetranitromethane increased with increasing pH under conditions in which the sulfhydryl groups were blocked with carbamyl phosphate. This finding is in accord with results on model compounds for which the rate of reaction seems to be proportional to the concentration of the ionized phenol. For proteins having tyrosyl residues with a p*K* near 10 (Bruice *et al.*, 1968; Sokolovsky *et al.*, 1966) the rate should change markedly from pH 9 to 7 and then more slowly at still lower pH values. After 1-hr reaction the total amount of nitrotyrosine produced as well as the total loss of enzymic activity decreased as the pH of the reaction mixture was lowered (see Table II). The formation of cross-linked dimer also was strongly dependent upon the pH at which the reaction was performed. As shown in Table II, no dimer was formed at pH 7 or below. Figure 1B shows a sedimentation pattern of nitrated material produced at pH 6.7. The protein migrated as a single boundary with a sedimentation coefficient of 5.9 S. When succinate, at a concentration sufficient to saturate the active site, was present in the reaction mixture during the nitration, the inactivation was decreased markedly and both the rate and extent of the nitration were reduced.

Limited Reaction with Tetranitromethane. The above studies on nitration of catalytic subunit indicated that carbamyl phosphate protects the sulfhydryl group, that low pH suppresses cross-linking and loss of enzymic activity and that succinate further decreases both the reactivity of tyrosine and the loss of enzymic activity. We therefore studied further the reaction at pH 6.7 in the presence of succinate and carbamyl phosphate by determining the number of tyrosyl and nitrotyrosyl residues by amino acid analyses of the hydrolyzed protein. As shown in Table III, the catalytic subunit contained 7.78 tyrosyl residues/polypeptide chain of mol wt 3.3×10^4. This result is in good agreement with the value of 8.5 obtained by Weber (1968a) and the value of 7.3 obtained by Changeux and Gerhart (1968). Reaction with tetranitromethane caused an almost linear increase with time in the number of nitrotyrosyl residues produced. However, as shown in column 3 of Table III, the total number of tyrosyl and nitrotyrosyl residues decreased 12% over a period of 4 hr. Under conditions recommended by Sokolovsky *et al.* (1966), Boesel and Carpenter (1970) found that tetranitromethane caused a loss of 75% of the total tyrosine and nitrotyrosine in glycyl-L-tyrosine. Similarly Vincent *et al.* (1970) found evidence for a loss in the total tyrosine plus nitrotyrosine in porcine trypsin under similar conditions. During the course of the reaction there was a progressive decrease in enzymic activity with time of reaction until about 50% was lost, followed by a period of 2 hr during which there was virtually no further loss of activity. If the reaction was terminated after 75 min a nitrated derivative was produced with an average of 0.70–0.85 nitrotyrosine/polypeptide chain. This derivative, which retained 75–90% of its original enzymic activity, was used for further studies.

Addition of dithionite to a sample of the modified protein caused an immediate loss of absorption above 350 nm, owing to reduction of the nitro group to an amino group (Sokolovsky

TABLE III: Nitration of Catalytic Subunit at pH 6.7.[a]

Time (hr)	Nitrotyrosine[b] (moles/mole)	Nitrotyrosine[b] + Tyrosine (moles/mole)	Sp[c] Act. (μmole/μg per hr)	Act. Remaining (%)
0	0	7.78	10.4	100
1	0.53	7.60	9.15	91
1.5	0.88	7.49	7.88	76
2	1.26	7.46	5.76	56
3	1.93	7.05	5.10	49
4	2.32	6.81	4.24	41

[a] Nitration was carried out at ambient temperature in 0.1 M potassium phosphate buffer (pH 6.7), containing 0.5 M KCl, 2×10^{-3} M EDTA, 0.02 M potassium succinate, and 0.004 M carbamyl phosphate. Tetranitromethane in ethanol was added to a final concentration of 0.01 M. The protein concentration was 4 mg/ml. [b] The number of nitrotyrosines and tyrosines per polypeptide chain (3.3×10^4 g) were determined by amino acid analysis corrected for loss during hydrolysis as described in Methods. [c] Specific activity in micromoles of carbamyl phosphate formed per hour were measured at 0.01 M aspartate.

TABLE IV: Kinetic Parameters for Native and Nitrated Catalytic Subunit.

pH	V_{max} (μmoles/μg per hr)		K_m (app, for Aspartate) (mM)	
	Native[a]	Nitrated[b]	Native[a]	Nitrated[b]
6.4	5.4	9.5	11	18
7.0	28	21	20	20
7.4	37	27	20	18
8.4	46	23	20	18

[a] Reprinted from Porter *et al.* (1969). [b] Nitrated material prepared at pH 6.7 contained 0.8 nitrotyrosine/polypeptide chain. Assays were performed in 0.2 M imidazole acetate buffer for pH 6.4 and 7, and in 0.2 M Tris-acetate buffer for pH 7.4 and 8.4 as described in Methods.

et al., 1966). The loss of absorption indicated that there was no substantial modification of tryptophan as was found by Cuatrecasas *et al.* (1968). Neither was there loss of histidine or methionine as judged by amino acid analysis. The nitrated catalytic subunit migrated as a single component on cellulose acetate electrophoresis and in the ultracentrifuge (Figure 1B) with an $s_{20,w}$ at 5 mg/ml of protein of 5.53 S. Under the assumption that the value for the concentration dependence of the sedimentation coefficient is the same for the nitrated derivative as for the native catalytic subunit (0.0089 ml/mg), the sedimentation coefficient at infinite dilution, $s^0_{20,w}$ was 5.79 S compared to 5.84 S for the native material (Kirschner and Schachman, 1971b). The nitrated derivative combined with regulatory subunit to form an enzyme-like complex with an $s_{20,w}$ of 11.0 S at 8 mg/ml; on the assumption that it possessed the same concentration dependence as that for native enzyme (0.009 ml/mg) the $s^0_{20,w}$ obtained was 11.7 S, a value identical with that of the native material (Gerhart and Schachman, 1965).[2] No attempt was made to characterize further the homogeneity of the derivative.

Kinetic Behavior of the Nitrated Derivative. Since the nitrotyrosyl residue has a pK near 7 (Riordan *et al.*, 1967), a sensitive way of determining whether nitration affected the enzymic activity of the catalytic subunit involved an examination of the kinetic parameters as a function of pH in the region where the nitrotyrosyl residue undergoes protonation and deprotonation. Table IV shows some results for the nitrated catalytic subunit along with the values for the native catalytic subunit obtained by Porter *et al.* (1969). The K_m for aspartate at saturating carbamyl phosphate concentration for the nitrated subunit and for the native subunit were similar and both were nearly independent of pH. The values for the V_{max} may not be exactly comparable since there is variation among different preparations of native catalytic subunit. However, both showed a similar dependence of V_{max} on pH except for values at pH 8.5.

Spectral Properties of the Nitrated Catalytic Subunit in the Absence and the Presence of Ligands. As Riordan *et al.* (1967) have shown nitration of *N*-acetyltyrosine produced a nitrotyrosyl residue with a pK of 7.0.[3] For the ionized phenol the absorption maximum was at 428 nm and the extinction coefficient was 4.10×10^3 cm^{-1} M^{-1}. The unionized phenol showed an absorption maximum at 360 nm with an extinction coefficient of 2.70×10^3 cm^{-1} M^{-1}. Their studies with carboxypeptidase indicated that the pK of the nitrotyrosyl residues in the protein, and hence in their spectra, varied with added ligands due apparently to changes in the local environment of the chromophore.

Figure 2 shows the absorption spectrum of the nitrated derivative at pH 7. The nitrotyrosyl residue has a maximum absorbance at 430 nm, with an extinction coefficient of 4.0×10^3 cm^{-1} M^{-1} and has an isosbestic point at 390 nm. The extinction coefficient at the isosbestic point is 2.8×10^3 cm^{-1} M^{-1} compared to 2.2×10^3 cm^{-1} M^{-1} for nitrotyrosine (Sokolovsky *et al.*, 1966). If 4.0×10^{-3} M carbamyl phosphate is added to the nitrated derivative, which is sufficient to saturate the enzyme, there was little change in the difference spectrum as shown in Figure 2. The addition of succinate at a concentration of 2.0×10^{-3} M and carbamyl phosphate at a concentration of 4.0×10^{-3} M caused a decrease of 14% in the absorbance at 430 nm and an increase in absorption at 360 nm with an isosbestic point of 390 nm as shown in Figure 2. Succinate alone has no effect on the spectrum. The pH of the solutions was carefully controlled to within 0.01 pH so that the addition of these ligands did not affect the final pH of the solutions. The decrease in absorption at 430 nm thus corresponds to a protonation of the nitrotyrosyl phenolic group caused by a shift to a higher pK.

Figure 3 demonstrates the shift in pK by titrating the nitrated catalytic subunit in the presence and absence of saturating concentrations of succinate and carbamyl phosphate. The pK of the unliganded species was found to be 6.25. In the presence of saturating succinate and carbamyl phosphate the pK was 6.62. In order to calculate the fraction

[2] The sedimentation velocity pattern of the reconstituted protein is shown in the following paper (Kirschner and Schachman, 1973).

[3] The pK of *N*-acetyl-3-nitrotyrosine was measured at an ionic strength of 0.7. Under conditions of lower salt the pK would be expected to be slightly higher.

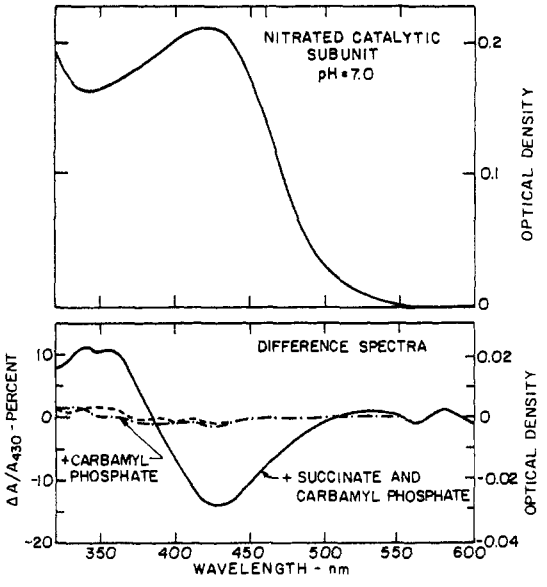

FIGURE 2: Spectrum and difference spectrum of nitrated catalytic subunit. The top drawing is the absorption spectrum of nitrated catalytic subunit containing 0.7 nitrotyrosine/polypeptide chain, at a concentration of 3 mg/ml in 0.04 M potassium phosphate buffer (pH 7). The bottom drawing shows difference spectra of the same sample. The protein *vs.* protein spectrum is represented by (- - - - -) which superimposes with the carbamyl phosphate, 2×10^{-3} M, difference spectrum represented by (—·—·—) above 450 nm. The solid line represents the difference spectrum obtained when succinate (2×10^{-3} M) and carbamyl phosphate (4×10^{-3} M) were added to one sample.

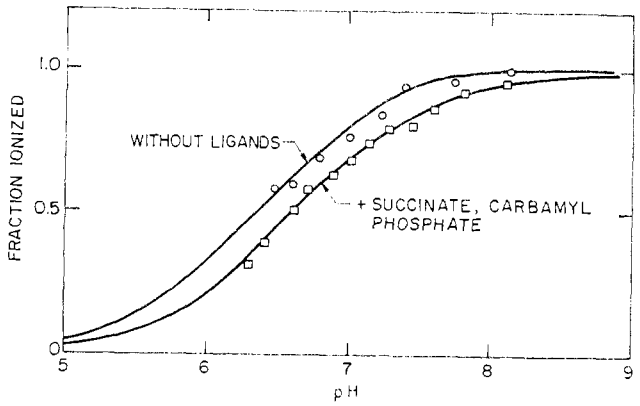

FIGURE 3: pH titration of nitrated catalytic subunit. The absorbance at 430 nm of nitrated catalytic subunit was measured in various phosphate buffers in the pH range of 6–8. One sample contained no added ligands and the other contained 2×10^{-3} M succinate and 4×10^{-3} M carbamyl phosphate. The fraction ionized is plotted *vs.* the pH, so that the p*K* corresponds to the half-ionization point on this graph.

ionized at each pH one requires the extinction coefficient at a specific wavelength, such as the maximum absorbance of the ionized form, both for the ionized and protonated form. Since the unliganded nitrated catalytic subunit precipitates below pH 6.3, it was impossible to measure the absorbance of the protonated form directly. For this calculation it was assumed that the absorbance at pH 8 at 430 nm represented the extinction coefficient of the ionized form. It was assumed further that the ratio of the absorbance at 430 nm of the protonated form of nitrotyrosine (measured at pH 5) to the ionized form (measured at pH 8.5) is the same for nitrotyrosine as it is for nitrated catalytic subunit. Small errors in this ratio, which was found to be 0.054, lead to negligible effects in the calculations.

Comparison of the Effects of Various Ligands on the Conformation of the Native Catalytic Subunit and on the Spectrum of Nitrated Catalytic Subunit. Various pairs of substrate analogs which seem to bind at the same site produce quantitatively different effects on the ultraviolet absorption spectrum of the catalytic subunit (Collins and Stark, 1969). Large changes were interpreted in terms of a conformational transition, while small changes were attributed to local effects at the binding site. Since the difference spectrum stems from binding of ligands at the active site, as well as from conformational changes in the protein, we have chosen to augment these studies with measurements of the ligand-promoted changes in the sedimentation coefficient. A comparison of a series of ligand pairs which bind to the same site but which produce different degrees of conformational changes should provide useful information as to whether the changes in spectrum of the nitrated derivative reflect binding or conformational transitions or both.

The ligand pair, succinate and carbamyl phosphate, binds with relatively high affinity to the catalytic subunit and permits reliable measurements of effects on difference sedimentation and difference spectroscopy. Other ligand pairs, however, which bind with much lower affinity pose special difficulties. In previous difference sedimentation experiments (Kirschner and Schachman, 1971a,b) ligands which were chemically similar but which bound with weaker affinity than the analogs to be tested were added to the reference sector to compensate for the contribution of the unbound ligand to the viscosity and density of the solution. If the affinity of the protein for the test ligand is high, so that most of it is bound, no compensating ligand is required in the reference solution. For experiments involving ligands of low affinities three problems develop. First, high concentrations of ligands are required and thus the viscosity and density contributions of the unbound ligands are no longer insignificant. Hence it is necessary either to use compensating ligands in the reference solution or estimate accurately the contribution of the unbound ligand to the viscosity and density of the test solution. Second, it is more difficult to find noninteracting reference ligands which are chemically similar to the test ligands. Third, at high speeds redistribution of the unbound ligand makes an observable contribution to the fringe pattern and the resulting curvature of the fringe pattern in the plateau and supernatant regions in the absence of a compensating effect in the reference solution causes errors in the base-line correction for difference sedimentation (Kirschner and Schachman, 1971a).

In spectroscopic experiments with the nitrated derivative, extreme care is needed to control the pH when high concentrations of ligands are added, since small shifts in the spectrum of nitrotyrosyl residues arise from a slight change in the pH of the solution. In addition very high concentrations of ligands would change appreciably the ionic strength of the solution causing a shift in the apparent p*K* of the group. For the above reasons we have chosen 0.02 M ligand concentrations for difference sedimentation studies and spectral studies with the nitrated derivative. Many substrate analogs at 0.02 M show an appreciable degree of saturation as determined by their inhibition constants and spectrally determined dissociation constants (Davies *et al.*, 1970; Collins and Stark, 1969). Ligands at this concentration would not measurably affect the p*K* of the nitrotyrosyl group in 0.04 M potassium phosphate buffer at pH 7 due to a specific effect of ionic strength. Phosphate buffer was employed since it has a small enthalpy of

ionization, and consequently there is only a slight temperature dependence of pH.

Table V shows the effect of some ligand pairs on the fractional change in the sedimentation coefficient, $\Delta s/\bar{s}$, of native and nitrated catalytic subunit. The control experiment with no ligands added to either sector gave a $\Delta s/\bar{s}$ of 0.003% corresponding to a change of 0.0002 S in the sedimentation coefficient. In expt 2, where the effect of glutarate was being tested, it was necessary to add a compound which would not interact with the protein but which would compensate to some extent for the viscosity and density contribution of glutarate and would also compensate for the redistribution of glutarate. Deoxyribose, though not a dicarboxylic acid, has an atomic composition and molecular weight similar to those of glutarate. However, it does not exactly mimic glutarate, since glutarate is present as a salt at pH 7. If it is assumed that deoxyribose makes the same contribution to the viscosity and density of the solution as glutarate, then from expt 2 it is seen that glutarate caused a 0.2% increase in the sedimentation coefficient. To test this assumption one should compare expt 3 and 4. If deoxyribose makes the same contribution to the viscosity and density of the solutions as the unbound aspartate, then aspartate caused a 0.48% increase in the sedimentation (expt 3). Since glutarate caused a 0.2% increase, then the net effect of glutarate in one sector and aspartate in the other should be 0.48 − 0.20 or 0.28%, a value close to the experimental result of 0.30%.

Though the experiments are internally consistent, the exact reference point may be slightly in error. For the experiments expressed relative to glutarate a value of 0.20% was added to the measured value of $\Delta s/\bar{s}$ (column 3) to obtain the value $(\Delta s/\bar{s})_{net}$ in column 4. This correction is based on the assumption that deoxyribose has no specific effects on the catalytic subunit and that deoxyribose compensates for the viscosity and density contribution of unbound glutarate. If there were no ligands in the reference solution to compensate for the unbound glutarate, $\Delta s/\bar{s}$ would have been −0.8%. Thus any error in the use of deoxyribose as an inert reference compound to simulate the ligand contributions to the viscosity and density of the solutions will affect the absolute values of $(\Delta s/\bar{s})_{net}$; however, the relative values for $(\Delta s/\bar{s})_{net}$ for the various ligands will not be affected.

Table V shows that L-aspartate, succinate, and L-malate (in the presence of phosphate[4]) caused a moderate increase in the sedimentation coefficient of about 0.4%. Since the ligand concentrations were 0.02 M we can estimate, from the dissociation constants found by Collins and Stark (1969), that the catalytic subunits were 40–80% saturated by the different ligands. When the ligands, L-malate and succinate, were present along with carbamyl phosphate, larger values of $\Delta s/\bar{s}$ were obtained (expt 7 and 8) with succinate and carbamyl phosphate producing the largest effect. As shown in expt 9 and 10 for the nitrated derivative, the effects of L-aspartate and phosphate and of succinate and carbamyl phosphate were similar to those for the unmodified catalytic subunit.

TABLE V: Effect of Various Ligands on the Sedimentation Coefficient of Native and Nitrated Catalytic Subunit.[a]

Ligands	$\Delta s/\bar{s}$ (%)	$(\Delta s/\bar{s})_{net}$[b] (%)
Native		
1. None/none	0.003	0.00
2. Glutarate/deoxyribose	0.20	0.20
3. L-Aspartate/deoxyribose	0.48	0.48
4. L-Aspartate/glutarate	0.30	0.50
5. Succinate/glutarate	0.18	0.38
6. L-Malate/glutarate	0.21	0.41
7. L-Malate, carbamyl-P/ glutarate, phosphate	0.57	0.77
8. Succinate, carbamyl-P/ glutarate, phosphate	1.40	1.60
Nitrated		
9. L-Aspartate/glutarate	0.44	0.64
10. Succinate, carbamyl-P/ glutarate, phosphate	1.07	1.27

[a] All experiments were performed at a temperature of 20 ± 0.5° in 0.04 M potassium phosphate buffer at pH 7 containing 2 mM mercaptoethanol and 0.2 mM EDTA. When carbamyl phosphate was used its concentration was 2 mM and the phosphate concentration in the reference sector was increased a corresponding amount. The concentration of all other ligands was 20 mM. The protein concentration varied between 5 and 8 mg per ml. [b] $(\Delta s/\bar{s})_{net}$ represents the values of $\Delta s/\bar{s}$ after correcting for the specific contribution of the ligands in the reference sector. It was assumed that deoxyribose had no specific effect.

Table VI compares the effects of a number of ligands on the conformation of the catalytic subunit as measured by changes in the sedimentation coefficient, differences in the ultraviolet absorption spectrum (Collins and Stark, 1969), and changes in the absorption of the nitrated catalytic subunit at 430 nm. Column 3 shows the fractional change of absorbance at 430 nm. The fourth, fifth, and sixth columns give the change in each parameter relative to that for succinate and carbamyl phosphate at a concentration of 0.02 M for the dicarboxylic acids. It is apparent from the results with all three methods that the effect of succinate and phosphate is small. The different methods all show that L-malate and carbamyl phosphate, L-asparate and phosphate produced intermediate effects of about 30–70% that found for succinate and carbamyl phosphate. D-Malate and carbamyl phosphate show substantial effects indicating agreement between the absorbance changes of the nitrated derivative and spectral changes in the near-ultraviolet region. Succinate and carbamyl phosphate show the largest effects by all of the methods. There is general qualitative agreement among the methods for all of the ligand pairs with the exception of L-malate and phosphate; for this pair of ligands Collins and Stark (1969) observed a large effect on the difference spectrum. Since these methods have different sensitivities to local and gross changes in the protein, a quantitative agreement is not expected.

Spectral Change of Nitrated Catalytic Subunit as a Function of Succinate Concentration. Figure 4 shows a plot of the per

[4] We have used 0.04 M phosphate buffer for these experiments in order to permit a comparison with the earlier results of Gerhart and Schachman (1968) and Kirschner and Schachman (1971b). Independent studies by Porter *et al.* (1969) and Kleppe (1966) have shown that phosphate is a competitive inhibitor of carbamyl phosphate. At concentrations of 0.04 M phosphate and 0.004 M carbamyl phosphate approximately 10% of the sites would be occupied by phosphate and 90% by carbamyl phosphate with less than 1% of the sites being unoccupied. Hence the observed effects are probably reduced by 10% compared to buffers containing no phosphate; however this effect should not alter the interpretation of the results.

TABLE VI: Effect of Various Ligands on the Conformation of the Catalytic Subunit.

(1) Ligands	(2) Concn (M)	(3) $(\Delta A_{430}/A_{430})^a$	(4) $(\Delta A/A)_{rel}{}^b$	(5) $(\Delta s/s)_{rel}{}^c$	(6) $(\Delta Uv)_{rel}{}^d$
Succinate	0.02	0.00	0.00	0.24	0.12
Succinate Phosphonacetamide	0.02 0.005	0.01	0.05		0.12
L-Malate	0.02	0.05	0.26	0.26	0.54
L-Malate Carbamyl-P	0.02 0.002	0.10	0.50	0.48	0.30
L-Aspartate	0.02	0.13	0.68	0.34	0.52
Malonate Phosphonacetamide	0.02 0.005	0.15	0.79		
D-Malate Carbamyl-P	0.02 0.002	0.18	0.95		0.65
Succinate Carbamyl-P	0.02 0.002	0.19	1.00	1.00	1.00

[a] The difference spectra measured at 430 nm were performed in 0.04 M potassium phosphate buffer at pH 7 containing 2 mM mercaptoethanol and 0.2 mM EDTA. [b] The data in column 4 represent values relative to that found for succinate and carbamyl phosphate. [c] The data obtained from Table V are expressed relative to the value for succinate and carbamyl phosphate. [d] The data of Collins and Stark (1969) obtained under slightly different conditions were recalculated for a ligand concentration of 0.02 M for the dicarboxylic acids. The concentration of phosphonacetamide was 0.005 M and of carbamyl phosphate was 4.9×10^{-3} M. Where not noted the other ligand was phosphate.

cent change in absorbance at 430 nm as a function of succinate concentration in the presence of a saturating level of carbamyl phosphate. At high succinate concentration the titration curve reached a plateau corresponding to a 15% change in absorbance. The dissociation constant obtained from a double-reciprocal plot of $(\Delta A_{430}/A_{430})^{-1}$ *vs.* (succinate concentration)$^{-1}$ is 3.7×10^{-4} M at pH 7 in 0.04 M potassium phosphate buffer 21°.

Inhibition of Catalytic Subunit by Succinate. As shown in Table VII, the inhibition constants, K_i, for succinate in the presence of carbamyl phosphate, are in the range of 0.77–0.97 mM at 30°; changing the ionic strength or the buffer ions had little effect. In contrast, the values of K_i at 20° in the same buffer (imidazole acetate and phosphate) were significantly lower, 0.29 and 0.38 mM. The latter values are in excellent agreement with the concentration of succinate required to produce a half-maximal change in the sedimentation coefficient (Kirschner and Schachman, 1971b) As shown in Table VII, even though phosphate inhibits the catalytic subunit by competitive binding with carbamyl phosphate (Porter *et al.*, 1969), it has little effect on the binding constant for succinate. Table VII also shows that K_m for aspartate is affected by buffer and temperature conditions in the same way as is the inhibition constant for succinate. Inhibition studies on the nitrated catalytic subunit gave the same value for K_i as found for the native catalytic subunit, indicating that the saturation curve obtained by measuring $\Delta A_{430}/A_{430}$ for the nitrated catalytic subunit (Figure 4) should be valid for the native catalytic subunit.

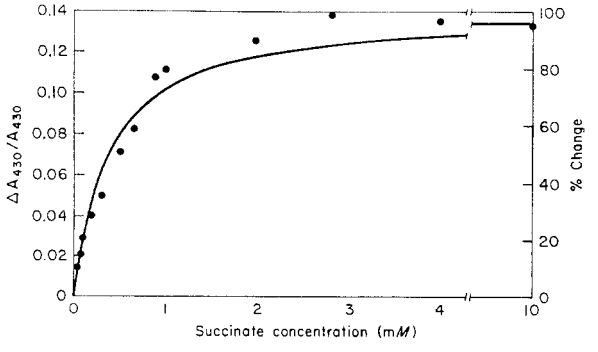

FIGURE 4: Succinate titration of nitrated catalytic subunit. The change in absorbance, $\Delta A_{430}/A_{430}$, where A_{430} is the initial absorbance at 430 nm, is given on the ordinate as a function of succinate concentration on the abscissa. All measurements were performed by difference spectroscopy. The nitrated catalytic subunit was at 4 mg/ml in 0.04 M potassium phosphate buffer at pH 7 containing 2 mM mercaptoethanol, 0.2 mM EDTA, and 4×10^{-3} M carbamyl phosphate. The solid line represents a theoretical curve for a single class of sites with a dissociation constant of 3.7×10^{-4} M.

TABLE VII: Inhibition of the Catalytic Subunit by Succinate.[a]

Buffer Concn (M)	Temp (°C)	K_i (mM)	K_m (mM)
Imidazole acetate (0.2)	30	0.97	13
Imidazole acetate (0.05)	30	0.77	10
Potassium phosphate (0.04)	30	0.82	11
Imidazole acetate (0.05)	20	0.29	6.0
Potassium phosphate (0.04)	20	0.38	6.6
Potassium phosphate (0.04)[b]	20	0.42	5.8

[a] Inhibition constants, K_i, were calculated from plots of the apparent K_m for aspartate *vs.* succinate concentration. For each of five concentrations of succinate, six concentrations of aspartate were used. Assays were performed in duplicate using the procedure of Porter *et al.* (1969). [b] This experiment was performed with nitrated catalytic subunit prepared as described in the text and assayed under the same conditions as native catalytic subunit.

Discussion

Under conditions originally suggested by Sokolovsky *et al.* (1966) and Riordan *et al.* (1967) for modification of tyrosyl residues in proteins, tetranitromethane has been shown to cause side reactions leading to modification of tryptophan (Cautrecasas *et al.*, 1968; Morihara and Nagami, 1969) and cross-linking probably of tyrosyl side chains (Doyle *et al.*, 1968; Boesel and Carpenter, 1970; Vincent *et al.*, 1970). It has been mentioned briefly (Riordan and Christen, 1968) that below pH 7 the potential side reactions of tetranitromethane with tryptophan, histidine, and methionine are abolished. In our study on the nitration of the catalytic subunit of aspartate transcarbamylase, even though it was not meant to be a systematic examination of the various effects of tetranitromethane, we have found that the amount of cross-linked material decreased strongly with decreasing pH. Even at pH 6.7, however, there were side reactions since the total amount of tyrosine and nitrotyrosine decreased progressively with the extent of nitration. The loss of total tyrosine and nitrotyrosine under these conditions was much less than that observed by Vincent *et al.* (1970) for bovine and porcine trypsins and trypsinogens or by Boesel and Carpenter (1970) for insulin and glycyl-L-tyrosine at pH 8. For catalytic subunit nitrated at pH 6.7 in the presence of succinate and carbamyl phosphate there was no evidence for any other modification of amino acid residues even though the slow rate of reaction at this pH required the use of high concentrations of tetranitromethane.

Of the eight tyrosyl residues per polypeptide chain in the catalytic subunit approximately two per chain react readily with tetranitromethane at pH 8. When the sulfhydryl groups are blocked by *p*-hydroxymercuribenzoate or by carbamyl phosphate, nitration leads to a product with residual enzymic activity when measured at pH 7. As shown in Tables II and III, slowing down the reaction by lowering the pH and adding saturating concentrations of succinate afforded some selectivity. Nitration of an average of one tyrosyl residue per chain occurs with little loss of enzymic activity; subsequent nitration of an average of two tyrosyl residues is accompanied by a substantial loss in activity. However, the separation of these two reactions is not complete and in the absence of information regarding modification of amino acid residues in particular polypeptide sequences it is not possible yet to ascertain how much reaction may have occurred at a second tyrosyl site. Limited nitration at pH 6.7 in the presence of ligands to the extent of 0.7–0.9 nitrotyrosine/polypeptide chain caused a loss of approximately 10–25% of the enzymic activity. The resulting protein was homogeneous in the ultracentrifuge and showed kinetic behavior similar to that of the native catalytic subunit.

The nitrotyrosyl derivative produced by limited nitration at pH 6.7 has an abnormally low pK of 6.25, compared to 7.2 for *o*-nitrophenol, 6.8 for 3-nitrotyrosine, and 7.0 for *N*-acetyl-3-nitrotyrosine (Riordan *et al.*, 1967). If the abnormally low pK of the nitrotyrosyl residues in the nitrated catalytic subunit is due to their local environment, we might expect the tyrosyl residues in the native protein to have an unusually low pK; such residues would be preferentially modified.[5] In both carboxypeptidase A and staphylococcal nuclease limited nitration produced derivatives with one modified tyrosine which had abnormally low pK's of 6.3 and 6.4, respectively (Riordan *et al.*, 1967; Cautrecasas *et al.*, 1968). In both cases addition of ligands caused an increase in pK as we observed for the nitrated catalytic subunit.

Addition of succinate and carbamyl phosphate to the nitrated catalytic subunit caused a decrease in absorbance (at 430 nm) of 14% which corresponds to a shift in pK from 6.30 to 6.74. The values for the pK measured by direct titration in the presence and absence of ligands were 6.25 and 6.62, respectively. It should be noted that experiments testing the effect of ligands on the spectrum of nitrated catalytic subunit were carried out at pH 7 to facilitate comparison with a large body of data obtained under those conditions and because the change in absorbance at that pH was sufficiently large to measure accurately. Had these experiments been conducted at a pH intermediate between the pK of the liganded and unliganded forms, *e.g.*, pH 6.4, then the spectral change would have been about 33%.

The low pK of the nitrotyrosyl group and the shift in pK on adding ligands merit interpretation for we would like to know the relationships among the spectral changes, the binding of ligands and the conformational changes in the catalytic subunit. Tyrosyl residues within a given protein and among proteins show wide variations in their pK's (Edsall and Wyman, 1958, chapter 9). Often tyrosyl residues have pK's higher than those for model compounds, and some are inaccessible to titration unless the protein is denatured (Edsall and Wyman, 1958, chapter 9; Tanford, 1962). Vicinal charged groups generally depress the pK's of the acids, *i.e.*, increase the ionization and, although the correlation is not perfect, there is a parallelism between the dipole moment of the substituent and the decrease in the pK of the acid (Edsall and Wyman, 1958). This is true whether the substituent has a positive or a negative charge, though the effect is more pronounced with positively charged groups. Carboxyl groups can substantially raise the pK of the phenolic group of tyrosine by specific hydrogen bonding. We might expect, therefore, that the particular nitrotyrosyl residues on the catalytic subunit, which have an abnormally low pK, are accessible to solvent and located in an environment near a high density of charged residues such as lysyl, arginyl, histidinyl, or carboxyl groups which are not in a position to hydrogen bond to the phenolic hydroxyl.

The effect of succinate binding is to raise the pK of the nitrotyrosyl group to a value more characteristic of model compounds. This could occur by a direct interaction of succinate with the nitrotyrosyl group or by a change in the local environment of that chromophore caused indirectly by the binding of succinate. Evidence as to which mechanism is correct will have to come from other sources. In particular one could argue that the effect is direct or indirect depending on whether one could show that the nitrotyrosyl group is located proximal or distal to the binding site for succinate.

If the nitrotyrosyl residue were located at the succinate binding site, its state of ionization would be expected to have an important influence on the binding succinate. In particular, if one of the succinate carboxyl groups interacted with the phenolic hydroxy group of the nitrotyrosine, then at a pH where the phenol was ionized this interaction would be unfavorable and the binding correspondingly weaker. Since succinate binds competitively with aspartate and at the same site (Porter *et al.*, 1969), the activity of the enzyme as a function of pH in the region of the nitrotyrosyl pK should be a sensitive measure of any direct interaction between succinate

[5] Recent studies (M. Bothwell and H. K. Schachman, unpublished observations) indicate that two tyrosyl residues per catalytic polypeptide chain titrate with an abnormally low pK about 9.0. Addition of succinate and carbamyl phosphate caused an increase in their pK just as shown here for the nitrotyrosyl residues in the nitrated catalytic subunit.

and the nitrotyrosyl group. As shown in Table IV, there is no evidence for any such effect. The K_m values for both native and nitrated catalytic subunit were virtually identical and characterized by their virtual lack of dependence on pH through the region of the protonation and deprotonation of the nitrotyrosyl residue. In addition, the maximum velocity showed an analogous pH dependence for the native and the nitrated derivative.

From studies with a number of ligand pairs it appears that the spectral change in the nitrotyrosyl chromophore occurs only with those ligands which produce conformational changes as measured by difference spectroscopy and difference sedimentation. Of these three techniques the spectral change in the nitrated catalytic subunit seems the most discriminating. Succinate and phosphate produced no observable spectral change at 430 nm. Succinate and phosphonacetamide, which produced a small difference change in the ultraviolet absorption spectrum characteristic of the binding of the ligands but not a conformational change (Collins and Stark, 1969), had little effect on the spectrum of nitrated catalytic subunit at 430 nm. In contrast, L-aspartate and phosphate, which form a kinetically identifiable dead-end complex with the enzyme (Porter *et al.*, 1969), and L-malate and carbamyl phosphate caused intermediate effects by difference sedimentation, difference ultraviolet spectroscopy and by the spectral change of the nitrated derivative at 430 nm. Whereas succinate and phosphate and succinate and phosphonacetamide appear to bind to the same site as succinate and carbamyl phosphate (Collins and Stark, 1969), the former have only small effects on the spectrum. This evidence suggests that mere binding of these ligands to the same site is not sufficient for a spectral change but that a conformational transition is also required if a large spectral change is to occur.

It appears that the spectral change of the nitrated catalytic subunit occurs only with those ligand combinations which produce conformational changes as measured by other methods and not with similar ligands which have been shown to bind to the same binding site. It is, however, still possible that the actual spectral change is produced by a direct effect of the bound dicarboxylic acid. For example, succinate in the presence of phosphate or phosphonacetamide may not be capable of binding in the proper orientation to interact with the nitrotyrosyl chromophore. In the presence of carbamyl phosphate a conformational change may occur which could place succinate in the proper configuration to interact with the chromophore. It is only the evidence that succinate does not protect the tyrosyl group from reaction with tetranitromethane, and that nitration of the catalytic subunit does not influence appreciably the enzymic activity that mitigates against this interpretation and in favor of an indirect effect transmitted by a conformational change. We would expect that if the nitrotyrosyl group were at the active site it would respond to either the binding of carbamyl phosphate or succinate.

The titration curve of the spectral change with succinate, shown in Figure 4, can be fit readily by a Michaelis–Menten-type of saturation function with a dissociation constant of 3.7×10^{-4} M. This value is similar to those obtained previously for succinate binding using other methods. A titration of a change in the optical rotatory dispersion by Pigiet (1971) gave a value for the half-titration point 2×10^{-4} M. The same value was obtained by difference sedimentation (Kirschner and Schachman, 1971b). As shown in Table VII, the value for the inhibition constant for succinate under the same buffer and temperature conditions was 3.8×10^{-4} M. The saturation curve obtained by measuring the change in the spectrum of the nitrated derivative as a function of succinate concentration in the presence of carbamyl phosphate is similar in form and gives a similar dissociation constant to those obtained by direct binding, by enzyme inhibition, or by other measures of conformational change. It seems, therefore, that binding is closely linked to conformational changes in the isolated catalytic subunit.

References

Babul, J., and Stellwagen, E. (1969), *Anal. Biochem. 28*, 216.

Boesel, R. W., and Carpenter, F. H. (1970), *Biochem. Biophys. Res. Commun. 38*, 678.

Bruice, T. C., Gregory, M. J., and Walters, S. L. (1968), *J. Amer. Chem. Soc. 90*, 1612.

Changeux, J.-P., and Gerhart, J. C. (1968), *Fed. Eur. Biochem. Soc., 4th Meeting, 1*, 13.

Collins, K. D., and Stark, G. R. (1969), *J. Biol. Chem. 244*, 1869.

Cuatrecasas, P., Fuchs, S., and Anfinsen, C. B. (1968), *J. Biol. Chem. 243*, 4787.

Davies, G. E., Vanaman, T. C., and Stark, G. R. (1970), *J. Biol. Chem. 245*, 1175.

Doyle, R. J., Bello, J., and Roholt, O. A. (1968), *Biochim. Biophys. Acta 154*, 457.

Edsall, J. T., and Wyman, J. (1958), Biophysical Chemistry, New York, N. Y., Academic Press.

Ellman, G. L. (1959), *Arch. Biochem. Biophys. 82*, 70.

Gerhart, J. C., and Pardee, A. B. (1962), *J. Biol. Chem. 237*, 891.

Gerhart, J. C., and Schachman, H. K. (1965), *Biochemistry 4*, 1054.

Gerhart, J. C., and Schachman, H. K. (1968), *Biochemistry 7*, 538.

Kirschner, M. W., and Schachman, H. K. (1971a), *Biochemistry 10*, 1900.

Kirschner, M. W., and Schachman, H. K. (1971b), *Biochemistry 10*, 1919.

Kirschner, M. W., and Schachman, H. K. (1973), *Biochemistry 12*, 2997.

Kleppe, K. (1966), *Biochim. Biophys. Acta 122*, 450.

Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951), *J. Biol. Chem. 193*, 265.

McClintock, D. K., and Markus, G. (1968), *J. Biol. Chem. 243*, 2855.

McClintock, D, K., and Markus, G. (1969), *J. Biol. Chem. 244*, 36.

Meighen, E. A., Pigiet, V., and Schachman, H. K. (1970), *Proc. Nat. Acad. Sci. U. S. 65*, 234.

Morihara, K., and Nagami, K. (1969), *J. Biochem. (Tokyo) 65*, 321.

Peterson, E. A., and Sober, H. A. (1962), *Methods Enzymol. 5*, 3.

Pigiet, V. P. (1971), Ph.D. Thesis, University of California, Berkeley, California.

Porter, R. W., Modebe, M. O., and Stark, G. R. (1969), *J. Biol. Chem. 244*, 1846.

Richards, E. G., and Schachman, H. K. (1959), *J. Phys. Chem. 63*, 1578.

Riordan, J. F., and Christen, P. (1968), *Biochemistry 7*, 1525.

Riordan, J. F., Sokolovsky, M., and Vallee, B. L. (1967), *Biochemistry 6*, 358.

Sedlak, J., and Lindsay, R. H. (1968), *Anal. Biochem. 25*, 192.

Shapiro, A. L., Viñuela, E., and Maizel, J. V., Jr. (1967), *Biochem. Biophys. Res. Commun. 28*, 815.
Sokolovsky, M., Harrell, D., and Riordan, J. F. (1969), *Biochemistry 8*, 4741.
Sokolovsky, M., Riordan, J. F., and Vallee, B. L. (1966), *Biochemistry 5*, 3583.
Svensson, H. (1950), *Acta Chem. Scand. 4*, 399.
Tanford, C. (1962), *Advan. Protein Chem. 17*, 69.
Vallee, B. L., and Riordan, J. F. (1969), *Annu. Rev. Biochem. 38*, 733.
Vanaman, T. C., and Stark, G. R. (1970), *J. Biol. Chem. 245*, 3565.
Vincent, J. P., Lazdunski, M., and DeLaage, M. (1970), *Eur. J. Biochem. 12*, 250.
Weber, K. (1968a), *Nature (London) 218*, 1116.
Weber, K. (1968b), *J. Biol. Chem. 243*, 543.
Weber, K., and Osborn, M. (1969), *J. Biol. Chem. 244*, 4406.

Local and Gross Conformational Changes in Aspartate Transcarbamylase†

Marc W. Kirschner‡ and H. K. Schachman*

ABSTRACT: Aspartate transcarbamylase and the free catalytic subunits isolated from it are known to undergo different, gross conformational changes upon the addition of the same, specific ligands. However, there has been little direct evidence indicating how, and to what extent, the catalytic subunits within the intact enzyme change upon the addition of ligands. In an effort to study the ligand-promoted conformational changes of the subunits within aspartate transcarbamylase we have prepared an enzyme-like complex composed of native regulatory subunits and modified catalytic subunits containing nitrotyrosyl chromophores whose spectrum was sensitive to the local environment. This modified enzyme exhibited the homotropic and heterotropic effects characteristic of native aspartate transcarbamylase. The spectral changes in the complex on binding ligands were similar to the changes observed for the free nitrated catalytic subunits. These spectral shifts representing local conformational changes were in marked contrast to the gross changes in hydrodynamic behavior which were in opposite directions for the intact enzyme and isolated catalytic subunit. The spectral change of the isolated subunit at varying succinate concentrations could be described by a hyperbola and was closely linked to the gross change in conformation revealed by difference sedimentation measurements. In contrast the enzyme-like complex had a titration curve for the spectral change which was sigmoidal in shape and which was clearly separated from the curve relating the change in sedimentation coefficient to succinate concentration. These results indicate that the local and gross conformational changes in the enzyme molecules are weakly linked. The local changes in the catalytic chains are sequential and strongly linked to ligand binding, whereas the gross conformational change appears to be concerted and is weakly linked to the binding of ligands.

Difference sedimentation experiments (Kirschner and Schachman, 1971a) have revealed that the free catalytic subunit of aspartate transcarbamylase undergoes a ligand-promoted conformational change opposite in direction to that exhibited by the native enzyme (Gerhart and Schachman, 1968; Kirschner and Schachman, 1971b). Whereas the isolated catalytic subunits change to a more compact or isometric conformation, the intact enzyme becomes more swollen or anisometric upon the binding of the same ligands. Although these hydrodynamic data show that the conformational change in the whole enzyme is not merely the sum of the changes observed for the isolated subunits, they provide virtually no information as to the relationship between the conformational changes in the isolated catalytic subunits and those for the same subunits within the enzyme complex. To investigate such a possible relationship we required a sensitive technique which could be employed selectively on both the isolated catalytic subunits and on these same subunits within intact enzyme molecules.

As shown in the preceding paper (Kirschner and Schachman, 1973), nitration of the catalytic subunit under controlled conditions produced a derivative having enzymic and physical properties similar to the native protein. Moreover, the nitrotyrosyl residues in the modified protein had an absorption spectrum which was altered markedly upon the addition of only those stereospecific ligands which promote conformational changes in the native subunit. This nitrated derivative combined with native regulatory subunits to form a reconstituted enzyme-like complex. Thus the nitrotyrosyl chromophore should serve as a sensitive spectral probe for the examination of conformational changes in the catalytic subunits both in the isolated state and within the intact enzyme molecules.

Gerhart and Schachman (1968) showed that the binding of succinate (in the presence of carbamyl phosphate) and the

† From the Department of Molecular Biology and the Virus Laboratory, Wendell M. Stanley Hall, University of California, Berkeley, California 94720. *Received October 25, 1972.* This research was supported by Public Health Service Research Grant GM 12159 from the National Institute of General Medical Sciences and by National Science Foundation Research Grant GB 4810X.

‡ Predoctoral Fellow of the National Science Foundation (1968–1971). This material was submitted in partial fulfillment of the Ph.D. requirements in Biochemistry at the University of California, Berkeley, Calif. Present address: Department of Biochemical Sciences, Princeton University, Princeton, N. J.