Acetylation of Human Serum Albumin by *p*-Nitrophenyl Acetate†

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ABSTRACT: Human serum albumin reacts very rapidly with *p*-nitrophenyl acetate (NphOAc). Rapid acetylation of the protein accompanies and largely accounts for the easily observed rapid formation of *p*-nitrophenolate ion. One group is acetylated much faster than all others. It appears to be located in a high affinity binding site for small fatty acid anions, to have a pK_a of 8.7, and a limiting bimolecular rate of reaction with NphOAc of $\sim 3 \times 10^4\ M^{-1}\ sec^{-1}$ at alkaline pH values. Rapid reversible binding appears to be a major contributor to the high reaction velocity.

Serum albumin reacts with *p*-nitrophenyl acetate[1] (NphOAc) much more rapidly than most proteins (Huggins and Lapides, 1947; Dirks and Boyer, 1951). The reaction, revealed by rapid formation of *p*-nitrophenol, appears obviously biphasic (Bruno and Ringold, 1969; Tildon and Ogilvie, 1969, 1972). Approximately 1 equiv of *p*-nitrophenol for each serum albumin appears to be formed very rapidly and is followed by slower formation of additional *p*-nitrophenol. Both phases of the reaction are significantly faster than the corresponding reaction of NphOAc with most proteins.

For bovine serum albumin the fast initial reaction with NphOAc has been attributed to an acetylation of one tyrosine residue of the slightly more than two that are acetylated by a 20-fold excess of NphOAc (Tildon and Ogilvie, 1972). Rate constants for the initial reaction have been reported for human (Bruno and Ringold, 1969) and bovine serum albumin (Tildon and Ogilvie, 1969) at different low pH values. The slower phase formation of *p*-nitrophenol has been studied in more detail and attributed to a catalytic hydrolysis of NphOAc (Tildon and Ogilvie, 1972).

We observed the reaction of NphOAc with human serum albumin and set out to determine the reason for the unusually high reactivity. The initial fast reaction is particularly interesting in that its rate at slightly alkaline pH after treatment to remove bound lipids (Chen, 1967) exceeds that for the analogous acylation of chymotrypsin by NphOAc (Hardman et al., 1971).

Experimental Procedures

Human serum albumin, crystalline (lot V 4414) from Mann Laboratories, crystallized human mercaptalbumin (lot 7), crystallized human serum albumin (lot 32) from Miles Laboratories, and crystallized human albumin from Sigma Chemical Co. were treated according to Chen (1967) to remove bound lipids. After treatment, rates of reaction of the different preparations with NphOAc differed by less than 5%. Recently recrystallized NphOAc provided by Dr. D. Griffiths was used as a stock solution dissolved in spectrograde acetonitrile. Labeled Nph[^{14}C]OAc was prepared from 150 mg of *p*-nitrophenol, 0.5 mCi of uniformly labeled [^{14}C]acetic anhydride (New England Nuclear), and 100 mg of unlabeled acetic anhydride in benzene and 2 equiv of pyridine. After washing and drying, the product was crystallized from a small volume of benzene to give a crystalline product with a melting point of 78–79°C and specific activity of 5.36×10^8 cpm/mmol of available *p*-nitrophenol. Hexanoic, octanoic, decanoic, myristic, and palmitic acids from Eastman Organic Chemical were used without further purification.

Counting of radioactive samples was done in 5 ml of 2,5-diphenyloxazole–1,4-bis[2-(5-phenyloxazolyl)]benzene in toluene plus 2.0 ml of 2-methoxyethanol in the presence of 50 μl of water with a Packard Tri-Carb liquid scintillation counter Model 314 EX-2.

Spectrophotometric measurements were made with a Cary 14 PM spectrophotometer with a thermostated cell holder at 25.4°C using either 0 to 1 or 0 to 0.1 Å slide wires. An extinction coefficient of 18000 $M^{-1}\ cm^{-1}$ at 400 nm was used to quantitate *p*-nitrophenolate anion in aqueous solutions above pH 8.5 (Kezdy and Bender, 1962). Concentrations of human serum albumin in aqueous solutions were determined by their absorption at 278 nm using an extinction coefficient $E_{278}^{0.1\%}$ of 0.531 (Kirschenbaum, 1968).

Reactions of NphOAc with serum albumin, unless noted otherwise, were followed by monitoring the appearance of absorption of *p*-nitrophenolate anion in the presence of a large excess of serum albumin. Reactions were initiated by rapidly mixing a solution of NphOAc in a few microliters of acetonitrile with buffered protein solution preincubated in the spectrophotometer by the use of a small plastic stirring rod ("plumper", Calatomic no. 85019) inserted through a small hole in the top of the cell compartment. With practice useful readings can be obtained in less than 3 sec. At pH values greater than nine, serum albumin concentrations were reduced to a tenfold excess over NphOAc in order to follow the very fast reactions. Rates of *p*-nitrophenolate ion formation in the absence of serum albumin were generally less than 1% of those in its presence and no corrections for such were made in those cases. Measurements of pH were made at the conclusion of each kinetic run and are relative to a pH 9.0 ± 0.01 standard buffer.

^{14}C-Acetyl-labeled serum albumin was prepared by reaction of serum albumin (69 mg) with 9.1×10^{-6} mol of

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[1] Abbreviations used are: HSA, human serum albumin; NphOAc, *p*-nitrophenyl acetate.

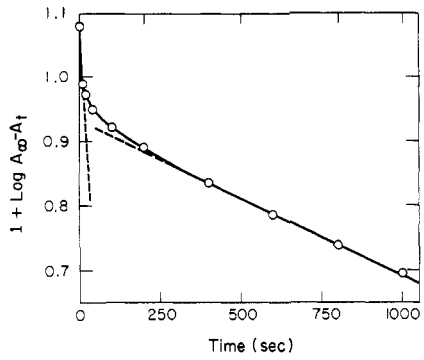

FIGURE 1: First-order plot for the reaction of excess NphOAc with human serum albumin. Monitored at 400 nm in 0.03 *M* triethanolamine-HCl buffer (pH 7.51), μ = 0.02 *M*, 0.5% acetonitrile, 25°C; [HSA] = 2.4×10^{-5} *M*, $[NphOAc]_{initial} \simeq 9.8 \times 10^{-5}$ *M*, $k_{initial} \simeq 2.05 \times 10^{-2}$ sec^{-1}, $k_{sec} = 5.43 \times 10^{-4}$ sec^{-1}.

Nph[^{14}C]OAc in 5.0 ml of 0.02 *M* triethanolamine-HCl, μ = 0.02, and 0.8% acetonitrile, and dialyzed overnight against 0.02 *M* acetic acid at 3°C.

Tryptic digestion of ^{14}C-acetylated serum albumin (10 mg/ml) after 5 min in a boiling water bath was done at pH 8 to 8.5 in 0.1 *M* triethylamine acetate buffer with 0.01 mg/ml of trypsin (Worthington L-1-tosylamido-2-phenylethyl chloromethyl ketone treated) for 2 hr at 37°C followed by an additional 0.01 mg/ml of trypsin for 6 hr. The reaction mixture was frozen and lyophilized. The sample was dissolved in 0.5 ml of water and centrifuged briefly and the clear supernatant applied to a 0.9 × 60 cm column of Sephadex G-15. The sample was eluted with 0.05 *M* trimethylamine–0.10 *M* acetic acid at a flow rate of approximately 0.2 ml/min and collected in 2.1- to 2.4-ml fractions.

Results

In aqueous solutions, in the absence of serum albumin, NphOAc undergoes slow hydrolysis as revealed by a slow appearance of *p*-nitrophenolate anion. Formation of *p*-nitrophenolate ion in such solutions is strongly enhanced by the addition of serum albumin (Dirks and Boyer, 1951). With excess NphOAc the formation of *p*-nitrophenolate is clearly not a continuous process (Bruno and Ringold, 1969; Tildon and Ogilvie, 1969, 1972). With relatively low molar excess the initial phase of the reaction as estimated from first-order plots by extrapolation to zero time, as shown in Figure 1, corresponds to approximately 1 equiv of *p*-nitrophenolate and is almost 40 times faster than the slower second phase.

Reaction with ^{14}C-Labeled NphOAc. The accelerated formation of *p*-nitrophenolate ion in the presence of serum albumin is not due to increased hydrolysis but largely reflects a rapid acetylation of serum albumin by NphOAc. Incubation with approximately a ninefold excess of ^{14}C-acetate-labeled NphOAc results, concomitant with complete formation of *p*-nitrophenolate ion, in the incorporation of approximately 5 equiv of label into the protein in a form not removable by extensive dialysis against unlabeled acetate (Table I). The overall reaction rate as reflected by appearance of *p*-nitrophenolate ion thus appears to correspond to the sum of a relatively large number of simultaneous reactions at different sites on the protein, S_1, S_2, S_3, . . . S_n plus hydrolysis according to the relationship shown in eq 1.

$$k_{obsd} = k_1[S_1] + k_2[S_2] + k_3[S_3] + \ldots k_n[S_n] + k_w \quad (1)$$

Table I: Acetylation of Human Serum Albumin by NphOAc.

NphOAc/HSA	Equiv Bound[c]
0.91[a]	0.87
1.3[a]	1.1
4.6[b]	2.9
9.2[b]	5.2

[a] [HSA] = 1.5×10^{-5} *M* in 0.02 *M* triethanolamine (pH 8.14), μ = 0.02, 3 hr at 24°C. [b] [HSA] = 9.5×10^{-5} *M* in 0.02 *M* triethanolamine (pH 7.5), μ = 0.02, 8 hr at 23°C. [c] After 36-hr dialysis against 0.02 *M* Tris–0.05 *M* acetate at 3°C.

With a ninefold excess of NphOAc hydrolysis accounts for less than half of the *p*-nitrophenolate ion formed. Most of the observed reaction appears to reflect stable acetylation of the protein. At lower NphOAc, hydrolysis accounts for an even smaller part of the observed reaction (see Table I). At very low levels, with the protein in excess, acetylation is very efficient and appears to account for all of the observed reaction.

A Fast Reacting Site. The initial rapid formation of *p*-nitrophenolate ion reflects the occurrence of a very fast reaction of NphOAc with serum albumin. This reaction, as indicated by the relative slopes in the first-order plot (Figure 1) of the initial and later parts of the reaction, is about 40 times faster than competing reactions. The amount of *p*-nitrophenolate ion formed in this part of the reaction corresponds very closely to the amount of serum albumin suggesting that only one fast reacting group is present. Very efficient incorporation of label with low levels of ^{14}C-acetyl-labeled NphOAc shows that the acetyl moiety becomes attached to the proteins in a relatively stable form during this reaction.

Evidence suggesting that only one group of the protein is acetylated at low levels of NphOAc is presented in Figure 2. After reaction with 0.9 equiv of ^{14}C-acetyl-labeled NphOAc, human serum albumin was digested with trypsin and the resulting peptides were passed through a column of Sephadex G-15. A large number of incompletely resolved peptides were observed to elute from the column by monitoring their absorbance at 254 nm (Figure 2). When the fractions were counted for radioactivity, however, more than 80% of the label applied to the column appeared in a single narrow zone apparently corresponding to a single peptide or possibly a few of very similar size. Most of the remaining label appeared in another, faster eluting, zone and might either indicate some labeling of another site or incomplete tryptic digestion.

Kinetics of the Fast Reaction. Monitoring the formation of *p*-nitrophenol or its anion affords a convenient means to follow the reaction of NphOAc with serum albumin. Rate constants for the initial reaction in the presence of excess NphOAc can be estimated from such data extrapolated to zero time. Under conditions such that the reaction is relatively slow one can obtain data sufficiently early in the reaction to reliably estimate the rate at zero time. Even so, this approach is somewhat inconvenient and subject to numerous possible inaccuracies. Uncertainty as to the concentration of NphAOc during the reaction and in the extinction coefficient of the product, *p*-nitrophenolate ion,[2] intro-

[2] *p*-Nitrophenolate ion is bound strongly by human serum albumin imparting a large blue shift in its absorption maximum (G. E. Means and M. L. Bender, unpublished results).

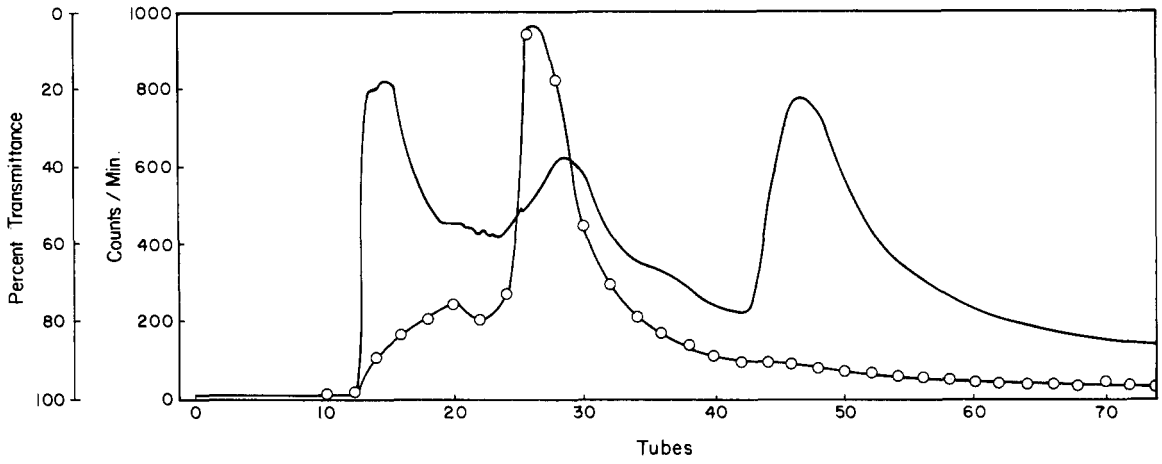

FIGURE 2: Elution profile of ^{14}C-acetylated serum albumin on Sephadex G-15 after tryptic digestion. Solid line is absorption at 254 nm; points are counts per minute for 50-μl aliquots. See Experimental Procedures for complete conditions.

duces a measure of error into such measurements. Under conditions such that reactions are very fast, rapidly changing rates do not permit reliable extrapolation to zero time.

To observe the fast reaction between NphOAc and serum albumin under a wide variety of conditions with greatest possible convenience and accuracy we have used the conditions such that serum albumin is in large excess as compared to NphOAc (i.e. HSA $\gg$ NphOAc). Under these pseudo-first order conditions reactions appear first order to the limit of detection and competing reactions do not seriously interfere.[3] Although relatively more of the more expensive reactant is required this does not represent a prohibitive cost in the present case. Other reaction conditions, for example, pH, ionic strength, temperature, or concentrations of serum albumin, NphOAc, or of various inhibitors, can be readily varied. Because reactions remain first order, rate constants can be readily obtained without extrapolation to zero time.

pH Dependence. Rates of reaction of NphOAc with serum albumin increase sharply with pH. Below pH 8 observed rates increase linearly with hydroxide ion concentration giving a slope of approximately 1.0 in a plot of log rate constant vs. pH (Figure 3). At higher pH values reaction rates increase more slowly and appear to approach a limiting value. The line drawn through the experimental points corresponds to a theoretical ionization profile for reaction of an unprotonated group with a pK_a of 8.7 and a limiting bimolecular rate constant of 3×10^4 M^{-1} sec^{-1}. Above pH 9 reaction velocities exceeding 0.4 sec^{-1} were obtained and the first 1 to 2 half-lives of the reaction were not observed and values generally reflect greater uncertainty as they are close to the limit of the spectrophotometric monitoring and sample mixing systems. The indicated limiting value of 3×10^4 M^{-1} sec^{-1} was supported by additional kinetic runs (not shown) at lower concentrations of serum albumin such that reactions were slower, more easily followed, and therefore more accurate but deviated from good pseudo-first-order conditions.

Binding of NphOAc. Substrate binding is thought to be an important feature of most enzyme-catalyzed reactions.

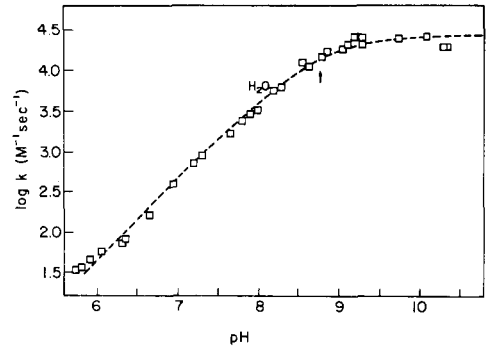

FIGURE 3: The reaction of NphOAc with serum albumin as a function of pH. Serum albumin in excess at 0.02 M ionic strength. Below pH 9 triethanolamine-HCl buffer with [HSA] = 5.1×10^{-5} M and above pH 9 N,N-dimethylethanolamine-HCl with [HSA] = 1.7×10^{-5} M. Arrow indicates the pK_a for calculated line assuming pK_a = 8.7 and $K_{obsd} = 3 \times 10^4$ M^{-1} sec^{-1}.

Binding may facilitate reactions, in part, by bringing reactants into close approximation (Bruice and Benkovic, 1966; Jencks, 1969; Bender, 1971). Binding of NphOAc by human serum albumin appears to be an important contributor to their subsequent rapid reaction. This binding can be represented by a simple equation:

$$\text{HSA} + \text{NphOAc} \overset{K_s}{\rightleftarrows} [\text{HSA} \cdot \text{NphOAc}] \overset{k_2}{\rightarrow} \text{acetyl-HSA} + p\text{-nitrophenol} \quad (2)$$

Rates of reaction thus increase with reactant concentrations but display saturation at high concentrations. With serum albumin in large excess this binding may be demonstrated and the corresponding dissociation constants, K_s, determined from a double reciprocal plot wherein the inverse of observed first-order rate constants is plotted vs. the inverse of the concentration of the reactant in excess, serum albumin (Figure 4). Values of K_s near $2\text{-}3 \times 10^{-4}$ M are obtained near neutral pH (see Table II). With NphOAc in excess, values of K_s were also determined but were done at relatively low pH in order to obtain data early in the reaction.

Inhibition by Fatty Acids. Commercial preparations of serum albumin generally include small amounts of strongly bound lipids. Fatty acids, frequently exceeding 1 equiv per albumin, and other bound lipids can be removed by treat-

[3] As expressed by eq 1 observed rates of p-nitrophenolate ion formation reflect the sum of many competing reactions. Because one reaction is at least 20× faster than the sum of all others, observed rates with serum albumin in excess largely (i.e. >95%) reflect the rate of that one reaction.

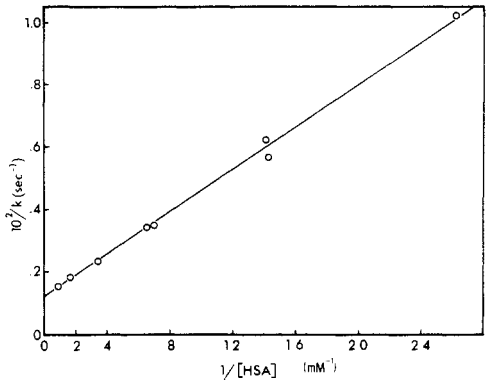

FIGURE 4: Double reciprocal plot for the reaction of NphOAc with serum albumin. Serum albumin in excess at pH 6.45 in 0.015 *M* NaH_2PO_4-0.005 *M* Na_2HPO_4 at 25.4°C.

Table II: Dissociation Constants and Maximum Velocities for the Reaction of NphOAc with Human Serum Albumin.

pH	$K_S \times 10^4$ *M*	k_2 (sec^{-1})
5.44[a]	6.6	
5.50[a]	8.2	
6.45[b]	2.8	0.0833
6.65[b]	3.3	0.0922
7.48[c]	3.3	0.239
7.48[c]	1.8	0.156
7.48[c]	1.9	0.148

[a] Mann lot V4414, 0.02 *M* sodium citrate, 10 ml/ml albumin, 25.4°C; from initial rates with NphOAc in excess. [b] Miles lot 7, 0.015 *M* NaH_2PO_4, 0.005 *M* Na_2HPO_4, 25.4°C; pseudo first order with albumin in excess. [c] Miles lot 32, 0.01 *M* triethanolamine, 0.02 *M* triethanolamine hydrochloride, 25.0°C; pseudo first order with albumin in excess.

ment at low pH with activated carbon (Chen, 1967). This treatment brings about a large (i.e., six- to tenfold) enhancement in the initial rate of reaction of serum albumin with NphOAc.

The addition of fatty acid anions to defatted serum albumin lowers its reactivity with NphOAc. Different fatty acids, however, differ considerably in their inhibitory capacity (Figure 5). Octanoate and decanoate are particularly potent inhibitors. Under conditions described in Figure 5, inhibition is almost complete with 1 equiv of either. Reactivity remaining in the presence of a slight excess appears to reflect the magnitude of competing reactions and is about 4% of that in the absence of an inhibitor. Other fatty acids examined were less effective but still relatively strong inhibitors.

Discussion

The reactivity of most proteins with NphOAc can be reasonably accounted for by their content of nucleophilic amino acid side-chain groups. Differences in reactivities of groups in these proteins, due to local environment effects, largely average out and overall reactivity is affected relatively little by denaturation. Thus, upon denaturation, ovalbumin, β-lactoglobulin (Dirks and Boyer, 1951), and sperm whale metmyoglobin (Breslow and Gurd, 1962) all experience slight increases in their reactivity with NphOAc. Serum albumin, by contrast, has an elevated initial reactivity and experiences a large decrease upon denaturation (Dirks and Boyer, 1951). Highly reactive groups in serum

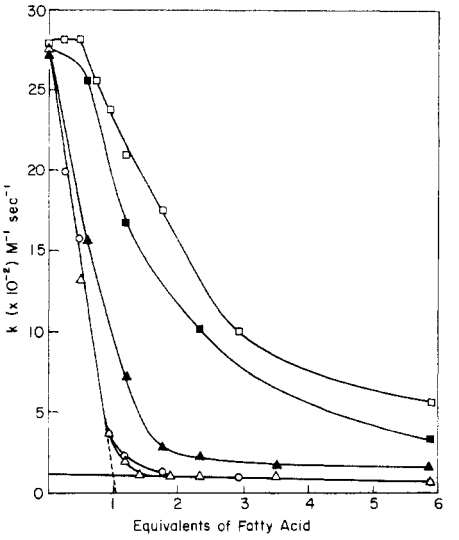

FIGURE 5: Inhibition of the reaction of NphOAc with serum albumin by hexanoate (▲); octanoate (○); decanoate (△); myristate (■); and palmitate (□). At pH 8.14, 0.03 *M* triethanolamine-HCl, ionic strength 0.02 *M*, serum albumin, 4.3×10^{-5} *M* at 25.4°C.

albumin appear to outnumber those with lower reactivity, presumably due to local environment effects about those groups and, in general, indicate something about the nature of the protein.

Groups in serum albumin probably react more or less independently with NphOAc at rates reflecting inherent chemical differences and different local environments. Because reactivities vary considerably, the formation of *p*-nitrophenolate ion appears discontinuous (Figure 1). With other proteins (Hartley and Kilby, 1954; Breslow and Gurd, 1962; Piszkiewicz and Bruice, 1968; Juliano et al., 1974) and model compounds (Bruice and Lapinski, 1958; Whitaker, 1962; Koltun et al., 1963) NphOAc reacts with amino, phenolate, sulfhydryl, and imidazole groups. Having a great many potential reactive groups, extensive acetylation of serum albumin should be possible with a sufficient excess of NphOAc. With only a ninefold excess five acetyl moieties are bound in a stable form, the remainder apparently undergoing hydrolysis. Acetylation of imidazole and sulfhydryl groups undoubtedly contributes to the formation of *p*-nitrophenolate ion but probably not the observed acetylation (Table I) as those acetyl moieties are relatively unstable. At relatively low levels of NphOAc, catalytic hydrolysis via these routes appears to be minor however, since stable acetylation accounts for most of the NphOAc and simple noncatalytic hydrolysis for much of the remainder.

Of the many reactions of NphOAc with serum albumin, one is of particular interest in that it is considerably faster than the sum of all others. With NphOAc in large excess the reaction of this group is seen as an initial fast release of 1 equiv of *p*-nitrophenolate ion superimposed upon slower formation of additional *p*-nitrophenolate ion (see Figure 1) and is accompanied by nearly quantitative attachment of an acetyl group to the protein (see Table I). The fast *p*-nitrophenolate ion formation thus represents a very fast acetylation of serum albumin by NphOAc.

To study the fast reaction of NphOAc with serum albumin with minimum interference from competing reactions we have used conditions wherein serum albumin is in large excess as compared to NphOAc. Under these conditions the fast reaction of this group accounts for most of the *p*-nitro-

phenolate ion formed and observed rate constants largely reflect its reaction rate elevated slightly by others occurring simultaneously according to eq 1 (i.e., see footnote 3). Under most conditions this added increment appears to be somewhat less than 5% as judged by both the relative rates of the two reaction phases in the presence of excess NphOAc (Figure 1) and the extent of inhibition by decanoate (Figure 5). The labeled acetyl group introduced upon reaction with labeled NphOAc under similar conditions appears to be associated primarily with a single tryptic peptide fraction.

Reaction rates generally increase with pH and, under the conditions given in Figure 3, approach a limiting rate of approximately $3 \times 10^4\ M^{-1}\ \text{sec}^{-1}$ at pH values above ten. The observed pH–log rate constant profile appears to correspond to that for the reaction of a basic group with a pK_a near 8.7. The apparent limiting rate at high pH is several orders of magnitude greater than expected for most simple nucleophiles of that pK_a and somewhat faster than observed for the corresponding active-site acetylation of chymotrypsin by NphOAc at its pH optimum (Hardman et al., 1971). The reaction is thus not merely a simple nucleophilic displacement but must be strongly facilitated by other features of the protein environment.

Strong reversible binding prior to reaction appears to be an important factor in promoting the reaction of NphOAc with serum albumin. Values of K_s, the NphOAc–serum albumin dissociation constants according to eq 2, obtained from double reciprocal plots (i.e., see Figure 4) range from approximately 8×10^{-4} to $2 \times 10^{-4}\ M$ from pH 5.45 to 7.48. As compared to similar simple intermolecular reactions this rather strong binding might account for several orders of magnitude increase in reactivity.

Bound lipids appear to have a strong inhibitor effect on the rapid reaction of NphOAc with serum albumin. With four commercial preparations of human serum albumin, treatment to remove bound lipids (Chen, 1967) brought about a six- to tenfold increase in reactivity with NphOAc. After such treatment all preparations were very similar. The observed increase varied, however, depending on the initial reactivity and the assay conditions. When fatty acid anions are added to fatty acid free serum albumin, its reactivity with NphOAc is decreased by as much as 97% depending on the particular fatty acid and its amount. The fast reaction with NphOAc thus appears to be associated with a fatty acid binding site of serum albumin.

Fatty acid anions of different chain lengths differ considerably in their inhibitory effects. Octanoate and decanoate in small stoichiometric excesses effect extensive inhibition. Longer and shorter chain-length fatty acids are less effective and greater excesses are necessary for comparable inhibition (see Figure 5). Assuming the inhibition results from simple reversible binding, corresponding dissociation constants for octanoate and decanoate appear to be less than $10^{-6}\ M$. Hexanoate is also a good inhibitor but appears to have a slightly higher dissociation constant. The reaction site thus appears to be associated with a primary binding site for small fatty acids, octanoate and decanoate in particular. The observed stoichiometry of the inhibition indicates the presence of only one such site in human serum albumin.

Larger fatty acid anions are less effective inhibitors of the reaction of serum albumin with NphOAc although considerable evidence suggests they are bound even more strongly (Teresi and Luck, 1952; Steinhardt et al., 1972). The inhibition by palmitate appears particularly interesting and may be instructive as to the basis for the weaker inhibitory effect of other large fatty acids. With less than 1 equiv of palmitate little or no inhibition is observed under conditions similar to those indicated in Figure 5. Slightly higher amounts lead to moderate inhibition. Preferential binding of palmitate to a site other than the reaction site may account for the observed effect. Saturation of that high affinity site with an equivalent of palmitate leaves subsequent equivalents free to interact with the reactive site.

The reaction of NphOAc with serum albumin appears to be like that of other acylating agents with other proteins but differs in one important respect. Reactivities of many groups in serum albumin appear to be significantly greater than normal. The individual reactions of these groups thus proceed much faster than expected and simple hydrolysis of the acylating agent is comparatively less. Reaction with the most reactive group appears to involve strong reversible binding prior to reaction. Binding of NphOAc adjacent to a nucleophilic group might account for the observed high reactivity and similar binding at other sites for the generally high reactivity. Strong binding of a wide spectrum of nonpolar molecules appears to be the single most outstanding feature of serum albumin. Its high reactivity with NphOAc may be a reflection of that binding capacity.

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Purification and Properties of a Ribosomal Protein Methylase from *Escherichia coli* Q13†

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ABSTRACT: Ribosomal protein methylase has been purified from *Escherichia coli* strain Q13 using methyl-deficient 50S subunits as substrates. The purified enzyme (or enzyme complex) which is devoid of rRNA methylating activity is quite stable and has a pH optimum around 8.0. The K_m for *S*-adenosyl-L-methionine is 3.2 μM. The molecular weight of the enzyme is 3.1×10^4; minor methylating activity was also detected for protein peaks with molecular weights of 1.7×10^4 and 5.6×10^4. Protein L11 is the major protein methylated by the purified enzyme. Product analysis revealed the presence of N^ϵ-trimethyllysine, a methylated neutral amino acid(s) previously observed in protein L11 and N^ϵ-monomethyllysine. Free ribosomal proteins were much better substrates for the methylation, indicating that methylation of 50S ribosomal proteins can occur before the complete assembly of the 50S ribosomal subunit.

It has been shown previously that several 50S ribosomal proteins contain methylated amino acids (Alix and Hayes, 1974; Chang et al., 1974; Chang and Chang, 1975). Protein L11 is the most heavily methylated of all the 50S proteins and contains a total of approximately five methyl groups per molecule of protein (Chang and Chang, 1975; Alix and Hayes, 1974). Three of these methyl groups are located in a single N^ϵ-trimethyllysine molecule with the remainder in one or more neutral amino acid(s) (Alix and Hayes, 1974; Chang and Chang, 1975).

Undermethylated 50S subunits can be obtained from an *Escherichia coli rel*$^-$ *met*$^-$ strain after starvation for methionine. Such 50S subunits can be methylated in vitro using crude enzymes derived from a high salt wash of unstarved *E. coli* Q13 70S ribosomes (Chang and Chang, 1974). It has been observed that more than 80% of the methyl groups incorporated in vitro were located in protein L11, and that the product was predominately N^ϵ-trimethyllysine (Chang and Chang, 1974). Protein L11 has been implicated in both the peptidyl transferase reaction during protein biosynthesis (Nierhaus and Montejo, 1973; Hsiung et al., 1974) and the binding of 50S antibiotics chloramphenicol (Nierhaus, 1974) and thiostrepton (Highland et al., 1975). Although enzyme(s) responsible for the methylation of lysine residues of cellular proteins from different mammalian tissues have been purified and studied (for review see Paik and Kim, 1975), no similar studies on ribosomal protein methylases, to our knowledge, have been carried out. In an attempt to elucidate the function of the methylation of ribosomal proteins, we have partially purified and characterized some of the properties of the methylating enzyme (or enzyme complex).

Materials and Methods

Bacterial Strains. Escherichia coli strain 1500, a *rel*$^-$ mutant of a K-12 strain, was obtained from Coli Genetic Stock Center in New Haven, Conn. The *met B* marker was introduced (strain 1500 *met*$^-$) by P1 transduction from JC-355 which is a K-12 *met*$^-$ strain originating with A. J. Clark. *E. coli* Q13 and A19 cells were purchased from General Biochemicals, Inc.

Materials. [*methyl*-^{3}H]-*S*-Adenosyl-L-methionine ([^{3}H]Ado-Met),[1] specific activity 8.5 mCi/μmol, in 0.01 *N* H_2SO_4–ethanol (9:1) and [*methyl*-^{14}C]-*S*-adenosyl-L-methionine ([^{14}C]Ado-Met), specific activity 55 Ci/mol, were obtained from New England Nuclear and Amersham/Searle, respectively. In most experiments, [^{3}H]Ado-Met was diluted to a specific activity of 1.0 mCi/μmol before use. Bovine serum albumin, ovalbumin, myoglobin, β-lactoglobulin A, and unlabeled *S*-adenosyl-L-methionine were from Sigma Chemical Co. Ultra pure ammonium sulfate was obtained from Schwarz/Mann. Omniflour was purchased from New England Nuclear. Other reagents were obtained from Sigma or local sources.

Preparation of the Substrates. Methyl-deficient 50S subunits derived from *E. coli* strain 1500 *met*$^-$ were prepared as described previously (Chang and Chang, 1974), and used in most experiments. In some experiments, dissociated proteins from 50S subunits were used. Dissociated 50S proteins were obtained by treating 50S subunits with an equal volume of 4 *M* LiCl and 8 *M* urea for 16 hr at 0°, followed by centrifugation at 15,000 rpm for 20 min to remove rRNA which was pelleted.

Determination of Protein Concentration. Protein concentration was determined according to the procedure of Schaffner and Weissmann (1973) using bovine serum albu-

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[1] Abbreviations used are: EDTA, ethylenediaminetetraacetate; Ado-Met, *S*-adenosyl-L-methionine; rRNA, ribosomal RNA.