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Purification and Properties of an Aminoglycoside Acetyltransferase from *Pseudomonas aeruginosa*†

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ABSTRACT: An aminoglycoside 3-acetyltransferase [AAC(3)], possibly a new isoenzymic species of the 3-*N*-acetyltransferase group, was purified to apparent homogeneity from a crude extract of *Pseudomonas aeruginosa*, a gentamicin-resistant clinical isolate. The method of purification was consecutive column chromatography—(i) gel filtration, (ii) affinity chromatography, and (iii) ion-exchange chromatography—to give two protein peaks, one of which was coincident with activity and which indicated a purification of 600 (specific activity = 9.743 units mg^{-1} at pH 7.2, 34 °C). Polyacrylamide disc gel electrophoresis indicated a single protein band coincident with enzymic activity. The molecular weight of the enzyme was about 39000. AAC(3)-V (provisonal designation) was further characterized by stability, substrate, pH, and kinetic studies. The K_m was 0.724 μM (sisomicin), and the V_{max} was 0.102 μmol min^{-1} mg^{-1} (sisomicin) at pH 7.2 and 34 °C. Substrate inhibition was exhibited by kanamycin A and tobramycin. Studies showed that enzyme activity was significantly stabilized when preparations contained substrate.

The major cause of aminoglycoside resistance in bacteria is now recognized as aminoglycoside-modifying enzymes (Davies & Smith, 1978; Devaud et al., 1977). Aminoglycoside antibiotics are inactivated by three enzyme-catalyzed transferase reactions—O-adenylylation, O-phosphorylation, and N-acetylation (Benveniste & Davies, 1973). *N*-Acetyltransferases modify aminoglycosides at the 6′- and 2′-amino positions of ring I (purpurosamine in gentamicin) and at the 3-amino position of ring II (deoxystreptamine). N-Acetylation on ring III (3-aminoglucose in kanamycin, tobramycin, and amikacin) is not known to occur (Figure 1). Hence, there are three acetyltransferase enzymes according to a classification indicated by the position of modification on the drug molecule (Haas & Dowding, 1975). In addition, there are subgroups of isoenzymes which modify the antibiotics at the same position but differ in their substrate profiles and in other biochemical properties. Thus, at present there are four isoenzymic forms of aminoglycoside 3-acetyltransferase (Davies & Smith, 1978).

This paper describes a chromatographic scheme for the purification of aminoglycoside 3-acetyltransferase from a crude extract of *Pseudomonas aeruginosa*, a clinical isolate resisant to kanamycin, gentamicin, tobramycin, and sisomicin. The method resulated in a 460-fold purification and gave a 23% recovery of the enzyme. Full characterization of the enzyme established that it was a new isoenzymic member of the 3-*N*-acetyltransferase group.

The degree of purity reported in this work is significant since many attempts to purify modifying enzymes have been unsuccessful, with one notable exception (Williams & Northrop, 1976), and have resulted in partial purifications only. The recovery of AAC(3)-V in high purity and activity was beneficial for kinetic and other studies, and for low background spectrophotometric assays, including serum gentamicin assay.

Materials and Methods

A gentamicin-resistant *Pseudomonas aeruginosa* culture was obtained as a clinical isolate from Sydney Hospital (July, 1977) after isolation with tryptone soy agar plates at 37 °C. Tryptone soy agar and broth were from Oxoid. CNBr–Sepharose 4B, Sephacryl S-200, and DEAE-Sephacel were obtained from Pharmacia. 5,5′-Dithiobis(2-nitrobenzoic acid) (DTNB), acetyl coenzyme A, kanamycin, and neomycin were purchased from Boehringer Mannheim. Low molecular weight calibration proteins for gel filtration were obtained from Sigma. Media for polyacrylamide disc gel electrophoresis was purchased from Eastman Organic Chemicals. Sisomicin and gentamicin were gifts from Schering Corp. (Essex Laboratories, Australia). Amikacin was a gift from Bristol-Myers (Australia), and tobramycin was a gift from Eli Lilly (Australia). All other reagents were of analytical grade.

Separation of the Kanamycin Components. Kanamycins A and B were separated from commercial kanamycin sulfate by the method of Inouye & Ogawa (1964). Kanamycins A and B were obtained as freeze-dried white translucent crystalline solids. The homogeneity of each component was established by paper chromatography.

Paper Chromatography of Kanamycins A and B. A descending paper chromatogram (Whatman no. 2 paper) was developed as described by Mason et al (1961). Kanamycin A (R_f 0.16–0.22) and kanamycin B (R_f 0.24–0.38) were de-

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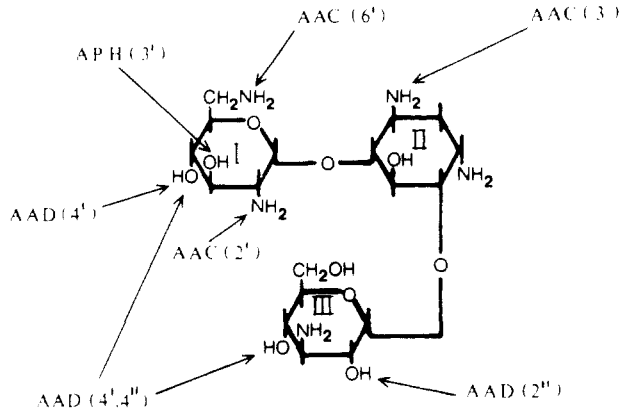

FIGURE 1: Kanamycin B: target sites for aminoglycoside-modifying enzymes.

tected as discrete ninhydrin-positive spots.

Separation of the Gentamicin C Components. Commercial gentamicin sulfate (1 g) was converted to the free-base form by ion-exchange chromatography on a Bio-Rex 70 (100–200 mesh, sodium form) column (2.4 × 16 cm). After the column was washed with distilled water, the gentamicin was eluted with 1% ammonia. The ninhydrin-positive fractions were pooled and rotary evaporated to remove ammonia and water. The residue (about 1 mL of a syrupy yellow liquid) was diluted with methanol (1 mL) and applied to preparative thick-layer silica gel G plates (20 × 40 cm) in a thin band by using a manual sliding applicator. The plates were developed by an ascending technique in the solvent system described by Wagman et al. (1968), the lower phase consisting of chloroform/methanol/16 N ammonia (2:1:1). After the solvent front had reached 15 cm from the origin, the plates were air-dried. A thin vertical band (0.5 cm) on each plate was sprayed with 0.1% ninhydrin in butanol in order to locate the three major gentamicin bands. The silica bands were scraped off the plates, extracted with 1% ammonia, and filtered. The clear filtrates were tested for homogeneity by thin-layer chromatography in the same solvent system. Gentamicins C1 and C1a (first and third bands) were completely separated, but gentamicin C2 (middle band) was contaminated with small amounts of C1 and C1a. Gentamicin C2 was chromatographed a second time and extracted as before. This second run produced essentially clean gentamicin C2. The freeze-dried, purified gentamicin C components were obtained as white translucent crystalline solids. The yields were the following: gentamicin C1, 48 mg; gentamicin C2, 27 mg; and gentamicin C1a, 36 mg.

The homogeneity of the separated gentamicins was confirmed by mass spectrometry. The potency of the separated components was tested by antibiotic bioassay and compared with a standard curve for gentamicin C complex. The melting points of the gentamicins were the following: gentamicin C1, 95–100 °C; gentamicin C2, 109–124 °C; the gentamicin C1a melting point was not estimated due to extreme hygroscopy.

Separation of the Neomycin Components. The method of Maehr & Schaffner (1964) was applied to the separation of commercial neomycin sulfate into the component species neomycins A, B, and C. The homogeneity of the separated species was tested by paper chromatography as described below.

Paper Chromatography of Neomycin Components. Neomycin complex and neomycins A, B, and C were run on a descending paper chromatogram (Whatman no. 2 paper) using the solvent system methyl ethyl ketone/*tert*-butyl alcohol/methanol/6.5 N ammonia (16:3:1:6) of Majumdar & Majumdar (1969). The chromatogram was developed for 40 h, air-dried, sprayed with 0.1% ninhydrin in butanol, and heated at 105 °C for 5 min.

Preparation of Crude Enzyme. Preparation of crude extract supernatant (S105) was largely as described previously (Haas & Dowding, 1975). Cells were grown in tryptone soy broth and were harvested in late log phase and then washed and disrupted (sonication) in buffer A [10 mM tris(hydroxymethyl)aminomethane hydrochloride (Tris-HCl) (pH 7.2) containing 10 mM $MgCl_2$, 0.15 mM ethylenediaminetetraacetic acid (EDTA), and 1 mM dithiothreitol]. Ultracentrifugation (105000*g*, 2 h, 4 °C) produced the S105 supernatant. Buffer B (buffer A without dithiothreitol) was used routinely for column chromatography and in all enzyme assays, since sulfhydryl reagents cause high blank backgrounds in the acetyl coenzyme A/DTNB coupled spectrophotometric assay.

Determination of Protein. Protein was measured by the method of Lowry et al. (1951) or by the ultraviolet absorption at 260 and 280 nm (Warburg & Christian, 1941).

Enzyme Assay. Acetyltransferase activity was measured by the DTNB spectrophotometric method (Alpers et al., 1965) modified according to Benveniste & Davies (1971a).

Assays of enzyme activity during purification and for substrate studies were made with a Varian Model 635 ultraviolet–visible (UV–vis) spectrophotometer and a Varian 9135 recorder. Full-scale optical densities of 0–0.1 were used. The thermostated cuvette compartment was maintained at 34 °C. All reactions were carried out in a total volume of 1 mL. The reference cell contained 500 μM DTNB (ϵ 15 700), 40 μM antibiotic, 10–100 μL of enzyme, 10 mM Tris-HCl (pH 7.2), 10 mM magnesium chloride, and 0.15 mM EDTA. The test cell contained the same as the reference cell plus 114 μM acetyl coenzyme A. The reaction was commenced by the addition of antibiotic, and the linear change in absorbance was followed at 412 nm. The assay was not limited by cofactor or substrate concentrations and was linear in the range of enzyme concentrations used. Enzyme activity was expressed in international units. High backgrounds due to an acetyl coenzyme A hydrolase and other proteins were absent during the latter stages of purification. pH profiles were determined as above by using purified enzyme and buffers of appropriate pH increment. Kinetic measurements were determined by using purified enzyme and appropriate serial concentrations of substrate antibiotics.

Preparation of Immobilized Kanamycin. Kanamycin–Sepharose 4B for affinity chromatography was prepared from CNBr-activated Sepharose as described elsewhere (Kawabe et al., 1975).

Electrophoresis. Polyacrylamide disc gel electrophoresis was performed by using gels containing 7% acrylamide and 0.18% *N,N'*-methylenebis(acrylamide), and Tris–glycine buffer (pH 8.3). Column fractions containing enzyme activity were pooled and dialyzed overnight (4 °C) against distilled water to remove salts. The volume of the sample was then reduced by packing the dialysis bag in Sephadex G200 (4 °C, four changes). When the volume had reduced to about 50 μL, it was combined with 25 μL of 40% sucrose containing 1% bromophenol blue.

Results

Purification of the Enzyme. Aminoglycoside 3-acetyltransferase in a crude S105 supernatant extract from *P. aeruginosa* was purified by successive passage through Sephacryl S-200, kanamycin–Sepharose, and DEAE-Sephacel. A flow chart for the purification steps is summarized in Table I.

Gel filtration through a Sephacryl S-200 column (1.6 × 80 cm; flow rate 15 mL/h in 6.7-mL fractions) was a stable and

Table I: Purification of AAC(3)-V from *P. aeruginosa*

procedure	volume (mL)	enzyme (units/mL)	enzyme (total units)	protein (mg/mL)	sp act. (units/mg of protein	yield (%)	x-fold purification
crude S105 supernatant	13	0.15	1.95	9.2	0.0163	100	1
Sephacryl S-200 fractions 13–15	20.1	0.085	1.71	0.324	0.262	88	16
kanamycin–Sepharose fractions 33, 34	9.0	0.160	1.44	0.033	4.85	74	298
DEAE-Sephacel fractions 10–12	8.1	0.055	0.45	0.0073	7.534	23	462
fraction 10 (best)	2.7	0.068	0.18	0.0070	9.714	9	596

reproducible process, even at room temperature. In this experiment, a 16-fold increase in enzyme purity and 88% recovery of activity were obtained in a single, nonspecific step. In actual fact, apparent recovery of enzyme activity was about 140%. The gel filtration profile was divided into two large, broad protein peaks, and the acetyltransferase activity peak was located on the trailing slope of the first protein peak.

Gel filtration fractions containing enzyme activity were combined and applied to a kanamycin–Sepharose 4B column (0.9 × 11 cm; flow rate 20 mL/h in 4.5-mL fractions) which was eluted with a NaCl gradient (0–1.2 M) in buffer B. The enzyme peak occurred at the base of the rising edge of the major protein band, in about 1 M NaCl. At this stage, the purification realized a 300-fold increase on the original crude extract. Attempts to release the strongly adsorbed enzyme from the affinity column with a pH gradient or by specific elution with substrate (sisomicin, 20 μg/mL) were unsuccessful. We concluded that the strong binding of the enzyme may be due to a composite effect of two parameters—the kanamycin-specific affinity and a nonspecific ion-exchange factor. Williams & Northrop (1976) reported contrasting results for a similar experiment—AAC(3)-I bound to gentamicin–agarose was released in a sharp peak by changing the pH of the eluent from 7.8 to 4.5, whereas elution with high ionic strength only produced a broad, dilute activity peak.

DEAE-Sephacel ion-exchange chromatography (0.9 × 10 cm bed; flow rate 20 mL/h in 2.7-mL fractions) of dialyzed affinity column fractions increased the specific activity of the enzyme from 4.85 to 9.7 units/mg. The sharp activity peak was located in a single, very small protein peak which eluted in about 0.75 M $(NH_4)_2SO_4$. A much larger protein peak appeared earlier in the profile (Figure 2).

Enzyme fractions from the ion-exchange column were dialyzed and concentrated. Subsequent polyacrylamide disc gel electrophoresis depicted a single protein band. A second, unstained gel was sliced, homogenized in buffer B, and assayed for enzyme activity. The acetyltransferase activity was located in the gel slice which corresponded with the stained protein band in the first gel (Figure 3).

Molecular Weight Determination. The molecular weight of the acetyltransferase was determined by gel filtration in Sephacryl S-200. The column was previously calibrated with standard globular proteins (molecular weights in parentheses), namely, cytochrome *c* (12700), α-chymotrypsinogen (24000), ovalbumin (45000), and bovine serum albumin (67000). The molecular weight of AAC(3)-V was estimated to be about 39000, which indicates that the enzyme is a small protein. This is consistent with the small molecular sizes of other aminoglycoside-modifying enzymes, ranging from 27000 for APH(3′)-III (Matsuhashi et al., 1975) to 63000 for AAC(3)-I (Williams & Northrop, 1976).

Substrate Studies. Elsewhere (George, 1980) chemical and physical analysis of the isolated and purified product (*N*-

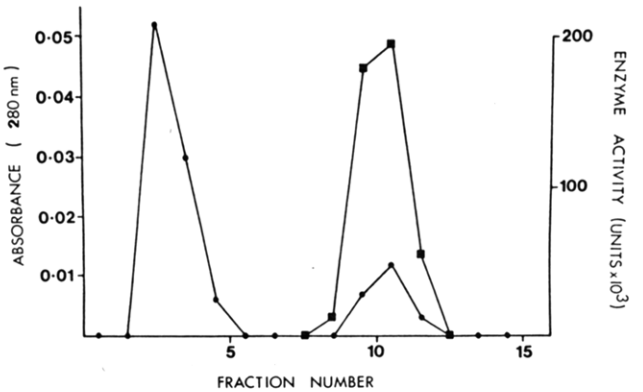

FIGURE 2: Ion-exchange chromatography of *Pseudomonas aeruginosa* AAC(3)-V (from affinity column) on DEAE-Sephacel. The plot represents protein (●) and AAC(3)-V activity (■). The enzyme was eluted in a stepwise ammonium sulfate gradient (0.15 M, 10 mL; 0.25 M, 10 mL; 0.75 M, 20 mL; and 1.00 M, 10 mL). The column was 0.9 × 11 cm and eluted at 10 mL/h in 2.7-mL fractions.

FIGURE 3: Polyacrylamide disc gel electrophoresis of purified AAC-(3)-V from *Pseudomonas aeruginosa*. The single protein band was coincident with acetyltransferase activity.

acetylsisomicin) of a reaction catalyzed by this acetyltransferase indicated that the 3-amino position on the deoxystreptamine ring was the target site for acylation of aminoglycoside antibiotics.

The full substrate profile of the purified enzyme is depicted in Table II. Sisomicin was by far the best substrate, followed by the closely related gentamicin C1a (sisomicin is 4′,5′-dehydrogentamicin C1a).

pH Profiles. The pHs for maximal enzymic reaction for several substrates of purified AAC(3)-V were measured in

Table II: Substrates for AAC(3)-V

substrate[a]	rel efficiency (%)[b]	substrate[a]	rel efficiency (%)[b]
sisomicin	100	amikacin	22
gentamicin C1a	53	kanamycin B	14
gentamicin C1	37	neomycin B	11
gentamicin C2	31	neomycin A	7
kanamycin A	26	neomycin C	6
tobramycin	22		

[a] Additional compounds which were tested but were found to be nonsubstrates included kasugamycin, streptomycin, streptamine, 2-deoxystreptamine, D-glucosamine, 3-*N*-acetylsisomicin, per-*N*-acetylkanamycin A, and per-*N*-acetylneomycin B. [b] Substrate efficiency represents the rate of inactivation of substrate per minute at 34 °C expressed as a percentage relative to that for the best substrate at pH 7.0. The substrate concentrations were 40 μM, and assays for enzyme activity were carried out by using the spectrophotometric procedure described under Materials and Methods.

Table III: K_m and V_{max} Estimates for Substrates of AAC(3)-V

		provisional estimates[c]		fine adjustment		SE[d]	
substrate[a]	$(r)^b$	K_m^0	V^0	K_m	V_{max}	K_m	V_{max}
sisomicin	0.9986	0.693	0.101	0.724	0.102	0.042	0.002
gentamicin C1	0.9983	1.608	0.046	1.453	0.045	0.101	0.001
kanamycin A	0.9892	2.611	0.087	5.358	0.102	0.571	0.008
tobramycin	0.9978	0.249	0.072	0.911	0.086	0.309	0.011

[a] Assays were performed at 34 °C and at the pH optimum of each substrate. Substrate concentration was varied by serial dilution in the range 0–40 μM. The enzyme concentration per assay was 0.005 IU. [b] (r) = linear correlation of experimental points. [c] K_m values are μM; V_{max} values are μmol min^{-1} mg^{-1}. [d] SE = standard error.

acetate (pH 4.0–6.5) and Tris-HCl (pH 7.1–9.0) buffers at 0.5 pH intervals. The enzyme was not catalytically active at pH 4 for any substrate, but activity was noted from pH 4.5 for all substrates and reached optimum values for neomycin B at pH 5.0–5.5, for sisomicin at pH 6.5, for gentamicin C2 at pH 8.0, for amikacin at pH 7.0, for the kanamycins at pH 7.3–8.0, and for tobramycin at pH 8.0.

Kinetic Parameters. Double-reciprocal Lineweaver–Burk plots were produced from acetyl coenzyme A/DTNB coupled spectrophotometric assays. The linear correlation of experimental points was generally excellent. Accurate measurement of initial velocities was enhanced by the sensitivity of the DTNB 412-nm assay and through the use of purified enzyme which virtually eliminated background absorbance. Statistical estimations (Wilkinson, 1961) of the kinetic parameters K_m and V_{max} of the Michaelis–Menton equation, and the relevant standard errors, are summarized in Table III. The first or provisional estimates of K_m and V_{max} were derived from the weighted fit of a linear regression. Fine adjustment of the provisional estimates was obtained from the fit of a bilinear regression of v (velocity) on the corresponding values of the provisionally fitted Michaelis–Menten function and its first derivative. Substrate inhibition was observed for kanamycin A (>5 μM) and tobramycin (>2.5 μM) in the Lineweaver–Burk plots, so that the K_m and V_{max} values listed for these two substrates in Table III are apparent values only. Sisomicin was the best substrate, and its low K_m reflected its high affinity for the enzyme. In fact, all substrates exhibited K_m values in the micromolar range and were acetylated rapidly. Very few accurate K_m and V_{max} estimates have been reported in the literature for aminoglycoside acetyltransferases, presumably due to the difficulty of obtaining highly purified enzyme preparations. However, the results in Table III for AAC(3)-V are consistent with the high substrate–enzyme affinities reported for other aminoglycoside-modifying enzymes (Holmes et al., 1974; Williams & Northrop, 1976; Yagisawa et al., 1975).

Stability Studies. A total of 97% of AAC(3)-V activity was salted out and recovered in a 70% saturated ammonium sulfate solution. Low concentrations of ammonium sulfate extended the storage stability of the enzyme in buffer A at 4 °C (22% recoverable activity after 4 months) or frozen at −20 °C (42% recoverable activity after 12 months). The recoverable activities were about 15% lower in the absence of ammonium sulfate. Purified AAC(3)-V was much less stable to freeze-drying than to freezing at −20 °C.

AAC(3)-V was maximally active in Tris-HCl and sodium acetate buffers, only 11% active in sodium cacodylate buffer, and inactive and unstable in sodium phosphate and Tris–maleate buffers. Absolute requirements of magnesium ions (10–40 mM) and a sulfhydryl reagent (dithiothreitol at 0.5–2.0 mM) were demonstrated for optimum activity and stability.

Discussion

The results presented in this paper indicate that the aminoglycoside acetyltransferase from a gentamicin-resistant *Pseudomonas aeruginosa* isolate is a new isoenzymic species of the 3-*N*-acetyltransferase group. This assignation is based on the evidence of substrate and kinetic studies and other properties of the enzyme and is consistent with the present convention of classifying aminoglycoside-modifying enzymes (Davies & Smith, 1978).

The substrate range of the enzyme was broad and differed from AAC(3)-I (Davies & Smith, 1978) and AAC(3)-II (Davies & Smith, 1978) in including amikacin, the kanamycins, and neomycins as substrates. The substrate profile indicated that this enzyme was nearer to the multisubstrate profiles of AAC(3)-III (Biddlecome et al., 1976) and AAC(3)-IV (Davies & O'Connor, 1978), but the differences in substrate efficiencies and the additional recognition of amikacin as a good substrate suggested that it was probably a new member of the AAC(3) group.

Substrate inhibition was observed for kanamycin A and tobramycin, and the Michaelis–Menten constants (for sisomicin) further illustrated the exclusivity of this enzyme's biochemistry. Also, other properties of the enzyme, namely, pH profiles, molecular weight, and stability characteristics, differed quite markedly from those of previously isolated acetyltransferases (Davis & Smith, 1978). Accordingly, it has been provisionally designated AAC(3)-V.

Mass spectral and [^{13}C]NMR studies of aminoglycosides modified by enzymes (Coombe & George, 1981) showed that in the acetylated product of the enzyme-catalyzed reaction between acetyl coenzyme A and aminoglycosides such as tobramycin, the 3-amino function of the deoxystreptamine moiety of tobramycin was in fact the target site of the transferase reaction (see Figure 1).

Partial purification of AAC(3)-V by gel filtration realized an apparent 140% recovery of enzyme activity. This anomaly was probably due to the inhibitory effect of contaminating proteins that had been removed during gel filtration of the crude extract, or to the presence of an acetyl-CoA hydrolase in the crude extract (Benveniste & Davies, 1971b), or both. In our experiment, background enzyme assay plots indicated that an acetyl-CoA hydrolase was present in the crude extract,

and this ancillary activity was eluted just before the acetyltransferase peak in the Sephacryl profile.

The stabilities of this and other aminoglycoside-modifying enzymes (R. G. Coombe and A. M. George, unpublished results) to gel filtration on Sephadex columns in which there is substantial loss of enzyme activity have been reported (Williams & Northrop, 1976; Umezawa et al., 1973).

In these experiments, the criteria for homogeneity of the enzyme were (a) elution of the purified enzyme from Sephacryl and DEAE-Sephacel columns in superimposed activity–protein peaks and (b) a single protein band coincident with acetyltransferase activity in acrylamide gel electrophoresis.

Stability studies and definition of buffer and ion requirements established limits for the successful purification of AAC(3)-V with maximal retention of activity, and for its subsequent employment in kinetic, pH, and substrate studies.

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Effects of Inositol Hexasulfate on the Oxygen Affinity of Hemoglobin: Verification of the Integral Function Theory of Thermodynamic Linkage†

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ABSTRACT: A detailed series of experimental measurements have been carried out to investigate the effects of inositol hexasulfate (IHS) on the oxygen binding curves of human hemoglobin. The data provide a critical test of the integral function theory for the mutual interaction of two ligands binding to a nondissociating macromolecule [Ackers, G. K. (1979) *Biochemistry 18*, 3372–3380]. This theory, which is required for cases where the fractions of bound and unbound ligands are of comparable magnitude, was found to predict quantitatively the observed effects. The experimentally determined variation of the median oxygen concentration with IHS concentration was analyzed by least-squares methods to determine the IHS binding constants for unliganded and fully oxygenated hemoglobin. The derived constants are in good agreement with independent estimates of their values, providing further verification of the theoretical treatment. General aspects of the integral function approach to thermodynamic linkage are briefly outlined. The importance of this approach for treating physiological situations is discussed.

In many physiological circumstances, as well as in vitro experimental situations, there is a need to understand the mutual influence of several small molecules in their binding reactions to a macromolecule. A frequently encountered case is that where the binding curve of one ligand, X, to a macromolecule, M, is influenced by the presence of a second ligand molecule, D, which also binds to M. Heterotropic effects in the regulation of allosteric proteins (Monod et al., 1965; Benesch & Benesch, 1967) are common examples of these phenomena.

Until recently there has been no theoretical treatment of this problem applicable to conditions where the concentrations of the "regulatory" molecule D in its bound and unbound forms are of comparable magnitude. This condition seems likely to be common under physiological circumstances and is certainly the case for in vitro experiments with regulatory

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