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SPECTROPHOTOMETRIC STUDIES OF BINARY AND TERNARY COMPLEXES OF OCTOPINE DEHYDROGENASE

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SUMMARY

1. The interaction of octopine dehydrogenase of *Pecten maximus* with coenzymes and substrate analogues was studied by a spectrophotometric difference method.

2. The coupling of the enzyme with coenzymes gives rise to spectral changes in the ultraviolet region similar to those given by a protonation of the adenine ring; in the visible region the difference spectrum presents a red shift for the reduced nicotinamide.

3. The binding of substrate analogues to the binary complexes produces a specific shift in the 288–300-nm region which should correspond to a red shift of the tryptophan residue spectrum. This specific shift is produced only by the substrate analogues that possess both the carboxylic and the guanidino groups.

4. The lack of interaction of arginine with apoenzyme is a possible direct evidence of an obligatory, ordered reaction sequence in enzymic reactions.

INTRODUCTION

Octopine dehydrogenase, a NAD^+ dehydrogenase which catalyses the dehydrogenation of octopine to give arginine *plus* pyruvate[1–4], has been recently purified from muscles of *Pecten maximus*[5]. Kinetic studies of the enzyme and its inhibition by substrate analogues suggest the existence of two binding sites on octopine dehydrogenase[5].

In the present work, a spectrophotometric difference method was used to investigate the interaction of the enzymic protein with the coenzymes, in presence or not of substrate analogues.

MATERIALS AND METHODS

NAD^+ and NADH (grade A), were purchased from Calbiochem. For NAD^+ the ε_{mM} was assumed to be 18.0 at 260 nm, and for NADH ε_{mM} was assumed to be 6.22 at 340 nm.

The substrates and the substrate analogues were commercial products, except

the guanidinobutane and δ-guanidinovaleric acid which were synthesized according to ref. 6, and octopine which was prepared by the method of IZUMIYA *et al.*[7].

Octopinic acid (α-*N*-(carboxy-1-ethyl)-L-ornithine)[8], homooctopine (α-*N*-(carboxy-1-ethyl)-L-homoarginine)[9], and nopaline (α-*N*-(1,3-dicarboxy-1-propyl)-L-arginine)[10] were kindly supplied by Professor G. Morel.

Enzyme preparation

Octopine dehydrogenase was prepared from muscles of *Pecten maximus* according to the method previously described[5].

Molecular weight

The molecular weight of octopine dehydrogenase was measured in 0.1 M phosphate buffer (pH 7) with 0.1 mM dithiothreitol by the high-speed sedimentation equilibrium method of Yphantis. Analyses were performed using ultraviolet optics. The molecular weight obtained was $36.5 \cdot 10^3$ (supposing $\bar{\nu} = 0.730$). The sedimentation coefficient $s^0_{20,w}$ was 3.4 S.

Enzymic activity

Enzymic activity was measured spectrophotometrically by determining the rate of NADH disappearance (or formation) at 340 nm as previously described[5].

Protein concentrations

Concentrations of proteins were calculated according to the method of WARBURG AND CHRISTIAN[11] or from the extinction at 280 nm by assuming that $E^{1\%}_{1\,cm} = 11.4$ determined by nitrogen analysis.

Spectral measurements

A Cary model 14 or 15 spectrophotometer fitted with a thermostatic cell holder was used to record difference spectra. The 0–0.1 slide-wire was used. The spectra were measured in two pairs of well-matched quartz cells with a path length of 0.4375 cm. The techniques described by LASKOWSKI *et al.*[12], and THEORELL AND YONETANI[13] were used. In the sample compartment, the front cell contained the enzyme in the buffer with the ligand and the rear cell contained the buffer alone. In the reference compartment, the front cell contained the enzyme in the buffer and the second cell contained the ligand in the buffer.

The enzyme was dialyzed, before spectrophotometric experiments, against 0.1 M phosphate buffer (pH 7.0) during 24 or 48 h, at 4°.

RESULTS

Binary complexes

Fig. 1A (Curve I) shows the difference spectrum between octopine dehydrogenase–NADH complex and its components. The ultraviolet region is characterized by a trough at 253 nm and a peak at 278 nm. Shoulders are also observed at about 286 nm and 300 nm. At longer wavelengths, a red shift occurs with a trough and a peak at about 325 and 375 nm respectively. This difference spectrum increases with increasing additions of coenzyme or enzyme (Figs. 2, 3).

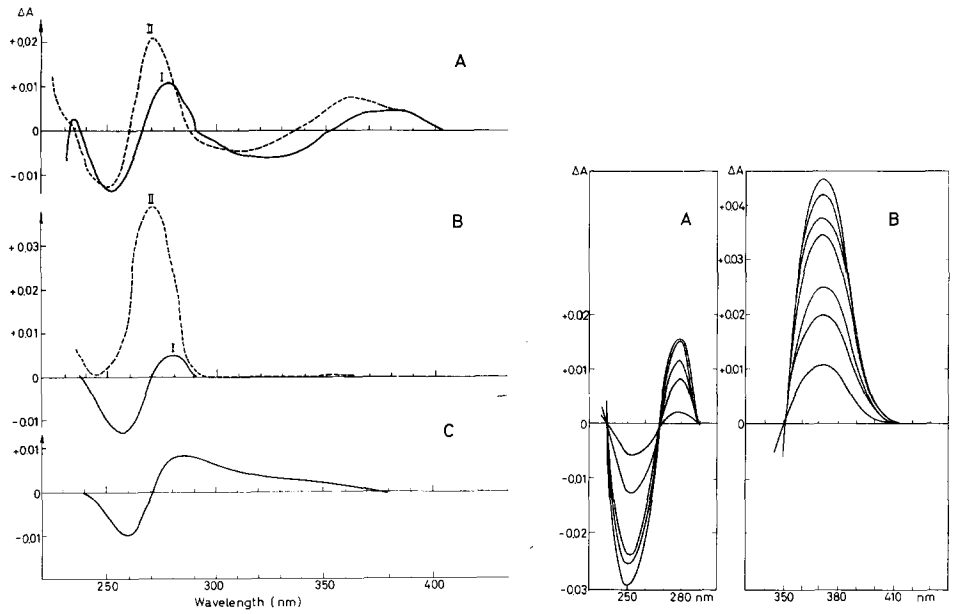

Fig. 1. A. Difference spectrum of octopine dehydrogenase–NADH complex. Curve I: protein 1.145 mg/ml, 0.187 mM NADH, 0.1 M phosphate buffer (pH 6.6). Curve II: 20% sucrose perturbation spectrum of 0.2 mM NADH. B. Difference spectrum of octopine dehydrogenase–NAD^+ complex. Curve I: protein 1 mg/ml, 0.14 mM NAD^+, 5 mM glycine–0.1 M phosphate–NaOH buffer (pH 9.8). Curve II: 20% sucrose perturbation spectrum of 0.2 mM NAD^+. C. Difference spectrum of octopine dehydrogenase–ADP complex. Protein 1.12 mg/ml, 0.22 mM ADP, 0.1 M phosphate buffer (pH 6.6).

Fig. 2. Difference spectra of octopine dehydrogenase–NADH binary complexes, with increasing amounts of coenzyme in 0.1 M phosphate buffer (pH 6.6). Temperature, 14°. A, in the ultraviolet region: protein 1.66 mg/ml; NADH 0.015, 0.0344, 0.057, 0.08 and 0.098 mM. B, in the visible region: protein 6.36 mg/ml; NADH 0.029, 0.0454, 0.0613, 0.081, 0.1, 0.119 and 0.137 mM.

The effect of the addition of NAD^+ to the apoenzyme (Fig. 1B, Curve I) is much less pronounced, and its study is hampered by the weaker affinity of the oxidized coenzyme and the great absorbance of enzyme and coenzyme in the region studied. Nevertheless, a red shift is observed with a trough and a peak at about 250 and 270 nm respectively. This effect increases also with increasing additions of coenzyme, but a saturation of the enzyme is rapidly obtained.

In order to elucidate the origin of these spectra we also measured the difference spectra resulting from the addition of ADP to the apoenzyme (Fig. 1C) and from the 20% sucrose perturbation spectrum of NADH and NAD^+ (Fig. 1A and B, Curves II)[14].

Sucrose perturbation of NADH gives approximately the same red shift as the addition of NADH to the apoenzyme (Fig. 1A). However, the shoulder at about 286 nm and the shift of the whole spectrum denote specific contributions from the protein.

Ternary complexes

Fig. 4A shows a significant alteration of the octopine dehydrogenase–NADH difference spectrum on addition of arginine—a substrate analogue— to the binary

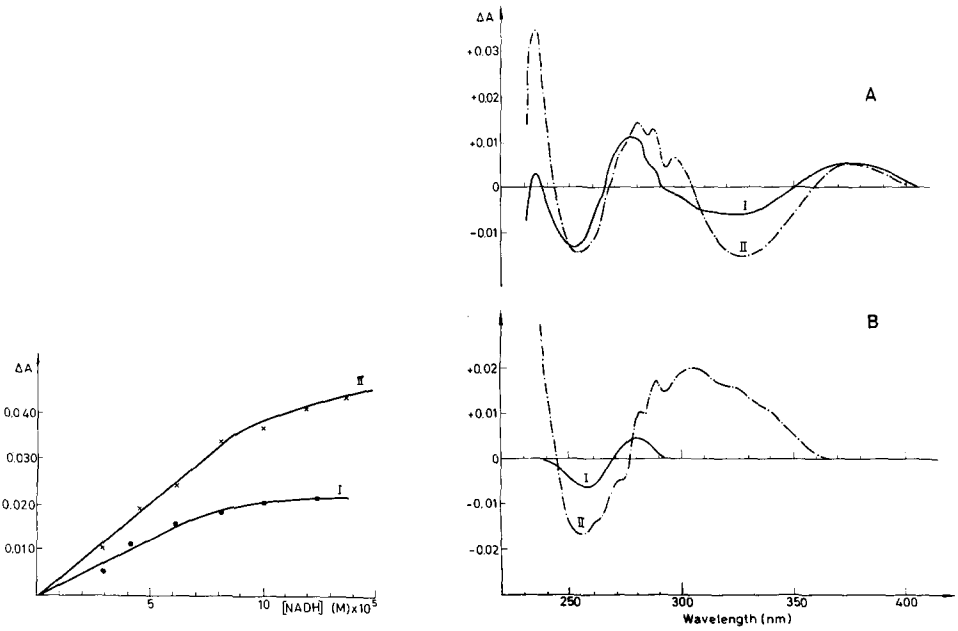

Fig. 3. Variation of the absorbance of octopine dehydrogenase–NADH complex at 375 nm in 0.1 M phosphate buffer (pH 6.6) at 14°. Curve I: protein 3.06 mg/ml. Curve II: protein 6.36 mg/ml.

Fig. 4. Interaction of arginine with binary apoenzyme-coenzyme complexes. A. Curve I, apoenzyme–NADH complex: protein 1.145 mg/ml, 0.167 mM NADH, 0.1 M phosphate buffer (pH 6.6). Curve II, the same: 4.4 mM arginine added. B. Curve I, apoenzyme–NAD+ complex: protein 1.16 mg/ml, 0.166 mM NAD+; 0.1 M phosphate–5 mM glycine–NaOH buffer (pH 9.8). Curve II, the same: 17.6 mM arginine added.

complex. Similar structural changes are observed when the NAD+–octopine dehydrogenase complex interacts with the same substrate analogue (Fig. 4B).

The main feature of the difference spectrum of the ternary complex *versus* the holoenzyme is the occurrence of two peaks in the 280–310-nm region, and the important peak around 238 nm (Fig. 5). The existence of the latter has been checked by lowering the apoenzyme concentration and extension of the wavelength region scanned to 235 nm.

Characteristic difference spectra were produced only by substrate analogues that possess both the carboxyl and guanidino groups, such as arginine (Fig. 5A and Fig. 6A), δ-guanidinovaleric acid (Fig. 5B and Fig. 6B) or octopine (Fig. 5C). Homooctopine induces a spectral effect of the same pattern as that produced by octopine, but of weaker intensity (Fig. 7). Arginic acid, which has an α-OH instead of an α-NH_2 group, is as effective as arginine, whereas D-arginine is not effective at all. Guanidinobutane and valerate, which are competitive inhibitors[5], octopinic acid, nopaline, pyruvate, L-lactate, ornithine, isoleucine, α-alanine as well as agmatine, do not give any significant spectrum.

Reaction order

Fig. 8 (A and B) shows that the interaction of arginine with apoenzyme or with

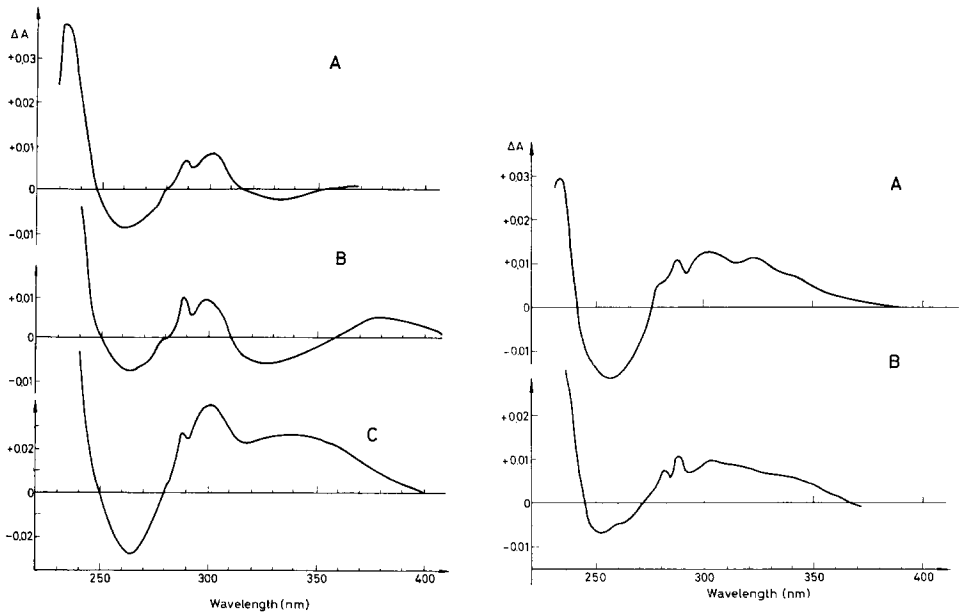

Fig. 5. Difference spectra of holoenzyme–substrate analogue complexes in 0.1 M phosphate buffer (pH 6.6). A: holoenzyme (protein 1.12 mg/ml *plus* 0.2 mM NADH), 13 mM arginine added. B: holoenzyme (protein 1.09 mg/ml *plus* 0.2 mM NADH), 0.45 mM guanidinovaleric acid added. C: holoenzyme (protein 1.05 mg/ml *plus* 0.2 mM NADH), 4 mM octopine added.

Fig. 6. Difference spectra of holoenzyme–substrate analogue complexes in 0.1 M phosphate–5 mM glycine–NaOH buffer (pH 9.8). A: holoenzyme (protein 1 mg/ml *plus* 0.2 mM NAD^+), 4.4 mM arginine added. B: holoenzyme (protein 1 mg/ml *plus* 0.2 mM NAD^+), 1 mM δ-guanidinovaleric acid added.

NADH or NAD^+ gives no signal; the characteristic spectrum is obtained only with the ternary complex.

DISCUSSION

Investigations of the ultraviolet absorption changes associated with enzyme–coenzyme complex formation show that some spectral modifications observed with octopine dehydrogenase are common to other NAD^+-linked enzymes[13,15].

The trough at 253 nm and the peak at 278 nm seem to be due to the interaction of the enzyme with the ADP moiety of the coenzyme. Actually the same red shift is obtained when ADP alone is added to the apoenzyme (Fig. 1C). On the other hand a good similarity is found between this spectrum and the pH difference spectrum of ADP-ribose (pH 6.5 *versus* pH 1.8)[16]. Interaction of the coenzyme with the enzymic protein gives rise to changes similar to those given by protonation of the adenine ring. RABIN suggests[16–18] that the protonation of adenine in alcohol dehydrogenase and ribonuclease may be due to the acidic form of lysine or arginine residues.

Blocking one lysine residue in arginine phosphotransferase and creatine phosphotransferase by dansyl chloride[19] prevented the formation of a peak at 278 nm after

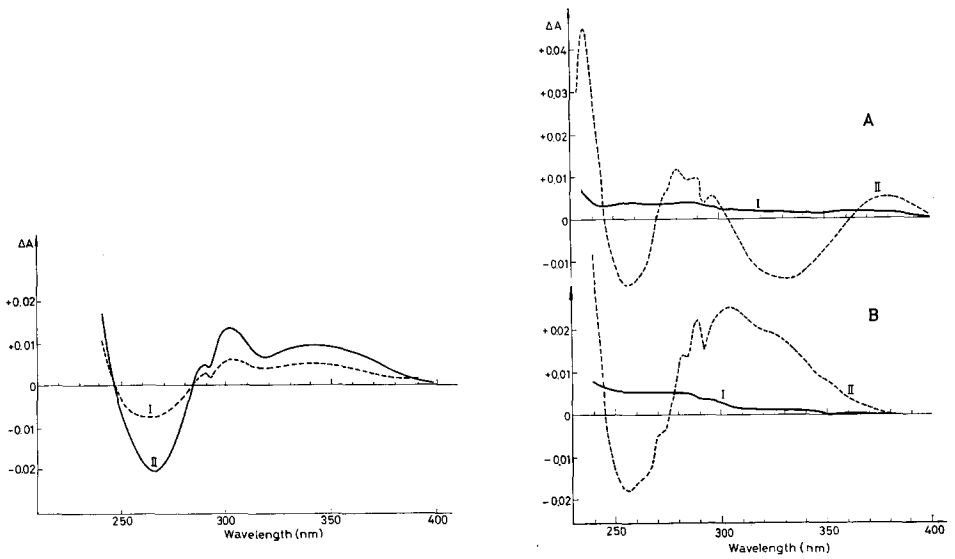

Fig. 7. Difference spectra of holoenzyme–homooctopine or octopine complexes in 0.1 M phosphate buffer (pH 6.6). Curve I: holoenzyme (protein 1 mg/ml *plus* 0.2 mM NADH), 6.4 mM homooctopine added. Curve II: the same, 2 mM octopine added.

Fig. 8. Reaction order. A: apoenzyme 1.16 mg/ml, 4.4 mM arginine added (Curve I) and then 0.167 mM NADH (Curve II) in 0.1 M phosphate buffer (pH 6.6). B. apoenzyme 1.16 mg/ml, 17.6 mM arginine added (Curve I) and then 0.166 mM NAD^+ (Curve II) in 0.1 M phosphate–5 mM glycine–NaOH buffer (pH 9.8).

addition of ADP to both enzymes. Since in our experiments the peak corresponding to the protonation of the adenine moiety appears even at a pH as high as 9.8, it is suggested that another residue in its acidic form, *e.g.* arginine, is involved in this protonation.

The shoulder at 286 nm exists neither in the adenine protonation difference spectrum nor in that due to sucrose perturbation of NADH or NAD^+. Thus this signal represents the specific contribution of the enzymic protein and could be due to a tryptophan residue; this signal is even more apparent in the ternary complex.

The reduced nicotinamide component of the binary complex spectrum resembles the sucrose perturbation difference spectrum of NADH (Fig. 1, Curve II). This differs markedly from the corresponding spectra of yeast alcohol dehydrogenase and lactate dehydrogenase, but resembles those of glutamate dehydrogenase–NADH or glutamate dehydrogenase–NADPH (refs. 15, 20). This red shift denotes some resemblance in the polarity of the coenzyme binding site of octopine dehydrogenase and glutamate dehydrogenase.

Attempts have been made to determine the number of NADH binding sites[21] on octopine dehydrogenase, using the signals at 253 and 375 nm (Figs. 2, 3). Although the peaks increase steadily with increasing additions of coenzyme, the results obtained were much lower than 1; this discrepancy is now under investigation.

It seems possible to identify the peaks at about 288 and 300 nm produced by the binding of substrate analogues to binary complexes as a red shift of the absorption of a tryptophan residue. Similar spectra were obtained by FISHER AND CROSS[22] with

glutamate dehydrogenase–NADH binding L-glutamate, and by HAYASHI *et al.*[23,24] with enzyme–substrate complexes of muramidase. In all these cases, the red shift shows that the substrate or substrate analogue fixation induces the moving of the tryptophan chromophore from the solvent-accessible region to a hydrophobic one.

SCHELLENBERG AND CHAN have demonstrated a direct participation of protein tryptophan residues in the hydrogen transfer catalysed by some dehydrogenases[25–27]. It would be interesting to study the role played by the tryptophan residue in the enzymic activity of octopine dehydrogenase.

Furthermore, the experiments with different substrate analogues indicate that only those having both the carboxylic and the guanidino groups contribute to the appearance of the tryptophan signal. It may be assumed that the binding of these two functional groups of the substrate to the enzymic protein induces a conformational change resulting in the exposure of a tryptophan residue.

The difference spectrum due to the interaction of arginine with the apoenzyme gives no signal; the tryptophan signal occurs only under conditions in which the protein–coenzyme–substrate (or analogue) ternary complex is formed. This is possible evidence from an obligatory ordered reaction sequence for the forward and reverse reactions catalysed by octopine dehydrogenase.

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REFERENCES

1 N. V. THOAI AND Y. ROBIN, *Biochim. Biophys. Acta*, 35 (1959) 446.
2 N. V. THOAI AND Y. ROBIN, *Biochim. Biophys. Acta*, 52 (1961) 221.
3 Y. ROBIN AND N. V. THOAI, *Biochim. Biophys. Acta*, 52 (1961) 233.
4 A. MENAGE AND G. MOREL, *Compt. Rend.*, 259 (1964) 4795.
5 N. V. THOAI, C. HUC, D. B. PHO AND A. OLOMUCKI, *Biochim. Biophys. Acta*, 191 (1969) 46.
6 E. SCHUTTE, *Z. Physiol. Chem.*, 279 (1943) 52.
7 N. IZUMIYA, R. WADE, M. WINITZ, M. C. OTEY, S. M. BIRNBAUM, R. J. KOEGEL AND J. P. GREENSTEIN, *J. Am. Chem. Soc.*, 79 (1957) 652.
8 A. MENAGE AND G. MOREL, *Compt. Rend.*, 261 (1965) 2001.
9 J. TEMPE AND G. MOREL, *Ann. Physiol. Végétale*, 8 (1966) 75.
10 A. GOLDMANN, D. W. THOMAS AND G. MOREL, *Compt. Rend.*, 268 (1969) 852.
11 O. WARBURG AND W. CHRISTIAN, *Biochem. Z.*, 310 (1941) 384.
12 M. LASKOWSKI, JR., S. J. LEACH AND H. A. SCHERAGA, *J. Am. Chem. Soc.*, 82 (1960) 571.
13 H. THEORELL AND T. YONETANI, *Arch. Biochem. Biophys.*, 106 (1964) 252.
14 T. T. HERSKOVITZ AND M. LASKOWSKI, *J. Biol. Chem.*, 237 (1962) 2481.
15 H. F. FISHER AND D. G. CROSS, *Science*, 153 (1966) 414.
16 R. F. FISHER, A. C. HAINE, A. P. MATHIAS AND B. R. RABIN, *Biochim. Biophys. Acta*, 139 (1967) 169.
17 S. TANIGUCHI, H. THEORELL AND A. AKESON, *Acta Chem. Scand.*, 21 (1967) 1903.
18 A. DEAVIN, R. FISHER, C. M. KEMP, A. P. MATHIAS AND B. R. RABIN, *European J. Biochem.*, 7 (1968) 21.
19 C. ROUSTAN, L. A. PRADEL, R. KASSAB, A. FATTOUM AND N. V. THOAI, *Biochim. Biophys. Acta*, in the press.
20 D. PANTALONI AND M. IWATSUBO, *Biochim. Biophys. Acta*, 132 (1967) 217.
21 A. S. STOCKELL, *J. Biol. Chem.*, 234 (1959) 1286.

22 H. F. FISHER AND D. G. CROSS, *Biochem. Biophys. Res., Commun.*, 20 (1965) 120.
23 K. HAYASHI, T. IMOTO AND M. FUNATSU, *J. Biochem. Tokyo*, 54 (1963) 381.
24 K. HAYASHI, T. IMOTO AND M. FUNATSU, *J. Biochem. Tokyo*, 55 (1964) 516.
25 K. A. SCHELLENBERG, *J. Biol. Chem.*, 240 (1965) 1165.
26 T. L. CHAN AND K. A. SCHELLENBERG, *J. Biol. Chem.*, 242 (1967) 1815.
27 T. L. CHAN AND K. A. SCHELLENBERG, *J. Biol. Chem.*, 243 (1968) 6284.