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A REEXAMINATION OF THE POSTULATED CHARGE TRANSFER INTERACTIONS AT THE ACTIVE SITE OF THE ENZYME RHODANESE

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Summary

Spectral and kinetic studies of the interaction of *N*-methylnicotinamide chloride and nicotinamide with the enzyme thiosulphate sulphurtransferase (thiosulphate : cyanide sulfurtransferase, EC 2.8.1.1) (also known as rhodanese) have been performed and compared with previous inhibition data obtained with *N*-1-(4-pyridyl)pyridinium chloride (NPP). Like NPP both *N*-methylnicotinamide chloride and nicotinamide are competitive inhibitors of rhodanese with respect to the substrate thiosulfate. Rhodanese binding of *N*-methylnicotinamide chloride gives rise to no charge transfer absorbtion band. In addition, the free energy of interaction (ΔG°) of NPP with rhodanese is approximately equal to the sum of the individual ΔG° values of MNA and NA. These compounds are analogous to the two halves of the NPP structure. We conclude that NPP and *N*-methylnicotinamide chloride are not bound via a charge transfer mechanism. The major stabilizing influence appears to be an ionic interaction with an anionic enzyme site with accessory apolar stabilization. It is postulated that the ionized active site sulfhydryl group in rhodanese could provide the ionic site.

Introduction

The conclusion that tryptophan residues are essential for the activity of the enzyme rhodanese (thiosulphate : cyanide sulfurtransferase, EC 2.8.1.1) has been inferred from the correlation between the *N*-bromosuccinimide [1] oxidation of this residue and the loss of enzymatic activity [1].

Further evidence for tryptophan being in the active site region came from kinetic inhibition studies using *N*-1-(4-pyridyl)pyridinium chloride. This compound belongs to a class of substances which act as electron acceptors and can form charge transfer complexes with indole derivatives [2,3]. Since NPP was a

Abbreviations: NPP, *N*-1-(4-pyridyl)pyridinium chloride; Tris, tris(hydroxymethyl)aminomethane.

competitive inhibitor of rhodanese with respect to thiosulfate, the interpretation was that the pyridinium compound competes with thiosulfate for the active site by virtue of its known affinity for the indole ring of tryptophan [1].

Wang and Volini [4] supported this idea by finding that aromatic sulfonates competitively inhibited the enzyme, and that their effectiveness roughly paralleled the electron accepting abilities of the compounds tested.

Studies also demonstrated that the transfer of sulfur to rhodanese resulted in a quenching of the intrinsic fluorescence of the enzyme in much the same way as elemental sulfur quenched the fluorescence of indole [1].

The mechanistic implications of these results were taken to be that tryptophan was directly involved in binding the substrate sulfur by virtue of a charge transfer interaction which involved transfer of charge from the π system of the indole ring to an overlapping non-bonding orbital of the sulfur atom [1].

Recent studies [5] have shown that concentrated solutions of rhodanese with bound substrate sulfur exhibit an absorbance shoulder in the area of 335 nm which disappears when the sulfur is removed with cyanide. This band was ascribed to an enzymic persulfide intermediate [6] involving the substrate sulfur and an ionized sulfhydryl group already shown to be an integral part of the active site [4]. Due to the spectral overlap between this new absorption band and the rhodanese emission it was proposed that the fluorescence quenching observed in the previous experiments occurred via a long-range energy transfer process [5].

This last result which does not require proximity between sulfur and indole taken together with the fact that *N*-bromosuccinimide can affect enzyme activity in ways other than simple tryptophan oxidation [7] raises questions about the direct involvement of this residue at the active site.

To gain further insight into this problem we have reexamined the charge transfer phenomenon using a combination of kinetic and spectral approaches.

Procedure and Results

Bovine liver rhodanese (M_r = 32 600) was isolated by the method of Horowitz and DeToma [8] with minor modifications. Crystalline enzyme was stored at —70°C as an ammonium sulfate suspension until used. *N*-Methylnicotinamide chloride, nicotinamide and *N*-acetyl-L-tryptophanamide were purchased from the Sigma Chemical Co. and used without further purifications.

Inhibition kinetics: *N*-methylnicotinamide chloride and nicotinamide were tested for their effects on the rhodanese-catalyzed reaction between cyanide and thiosulfate. The inhibition assay system was identical to the assay system for rhodanese activity as described by Wang and Volini [9] with two exceptions. The thiosulfate concentration was varied from 1—5 mM rather than the usual constant 50 mM level. When the inhibition by *N*-methylnicotinamide chloride was studied, the assay solution was 14 mM in this compound and when nicotinamide was the inhibitor being studied, the solution was 155 mM in this derivative. The control contained no inhibitor. Each assay contained 2.7 micrograms of enzyme. At each thiosulfate concentration, the assay was performed at room temperature and stopped at intervals of 15, 30, 45 and 60 s. The initial velocity (v_i) of the reaction was determined from the linear portion

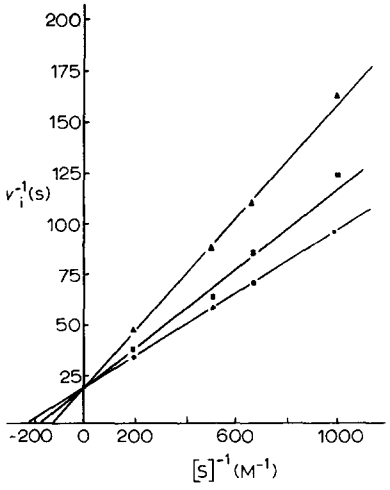


Fig. 1. Inhibition of rhodanese with *N*-methylnicotinamide chloride and nicotinamide. Ordinate, reciprocal of the initial reaction velocity; Abscissa, reciprocal of the molar inhibitor concentration. Lower line (●) contained no inhibitor; upper line (▲) contained 14 mM *N*-methylnicotinamide chloride; middle line (■) contained 155 mM nicotinamide. The assay system is detailed in the text.

of a plot of the optical density at 460 nm versus time. The data was analyzed by the Lineweaver-Burk formula. See Fig. 1 for the experimental data. Values of the inhibition constants, K_I, for *N*-methylnicotinamide chloride and nicotinamide are given in Table I along with the K_I for NPP reported previously [1].

Charge transfer titration: To determine whether the binding of *N*-methylnicotinamide chloride was due to a charge transfer interaction, both the sulfur

TABLE I

BINDING PARAMETERS OF *N*-METHYLNICOTINAMIDE CHLORIDE TO RHODANESE AND *N*-ACETYL-L-TRYPTOPHANAMIDE

Rhodanese	MNA [a]	NA [a]	NPP [a]
Type of inhibition:	Competitive	Competitive	Competitive
K_I [b]	$1.54 \cdot 10^{-2}$	0.44	$5.0 \cdot 10^{-3}$
K_{ASSOC} [c]	64.9	2.3	200

	Complex formation constant (k) M^{-1}	Complex molar extinction coefficient (ϵ) ($M^{-1} \cdot cm^{-1}$)
Skatole [d]	4.4 ± 0.2	1720
3-Indolacetic acid [d]	4.4 ± 0.2	1440
N-Acetyl-L-tryptophan [d]	4.5 ± 0.2	1330
3-Indolacetamide [d]	3.4 ± 0.2	1320
N-Acetyl-L-tryptophanamide [d]	4.0 ± 0.2	1180
N-Acetyl-L-tryptophanamide (This work)	3.6 ± 0.1	1035

[a] Abbreviations, *N*-methylnicotinamide chloride (MNA); nicotinamide (NA); *N*-1-(4-pyridyl)pyridinium chloride (NPP)

[b] See text for the evaluation of K_I.

[c] $K_{ASSOC} = (K_I)^{-1}$

[d] See ref. 11.

free ($1.95 \cdot 10^{-4}$ M) and sulfur substituted ($1.5 \cdot 10^{-4}$ M) forms of rhodanese in 0.2 M Tris, pH 7.9 (HCl) were titrated with *N*-methylnicotinamide chloride by the method of Deranleau et al. [10]. Absorbances were measured using a Cary 14 spectrophotometer equipped with a 0—0.1 OD slide wire. The total concentration of *N*-methylnicotinamide chloride varied from 0.07 M—0.82 M. Volume deviations upon addition of the solid compound were ignored.

The sulfur substituted form of the enzyme for the above titration was prepared by dissolving crystalline rhodanese in 0.20 M Tris, 8.5 mM $Na_2S_2O_3$, pH 7.9 (HCl) and centrifuging to remove any insoluble material. This sample was applied to a Bio-Rad P-2 column (0.9 × 15 cm) and eluted with 0.20 M Tris, pH 7.9 (HCl). Rhodanese containing fractions were combined to yield an enzyme concentration of $1.5 \cdot 10^{-4}$ M.

To prepare the sulfur free form of the enzyme, 0.2 ml of 0.083 M KCN, 0.1 M KH_2PO_4, pH 7.2 was added to 1.0 ml of a clarified solution prepared by dissolving crystalline rhodanese in 0.20 M Tris, pH 7.9 (HCl) and centrifuging. After a 10 min incubation the solution was desalted on a P-2 column as described above. The combined rhodanese fractions was $1.95 \cdot 10^{-4}$ M via a biuret assay.

Data from the titration of the two rhodanese forms were treated using the analysis described by Deranleau and Schwyzer [11]. Fig. 2 shows the data presented in the form of a Scatchard plot: $A/[P_0][X_0]$ versus $A/[P_0]$, where

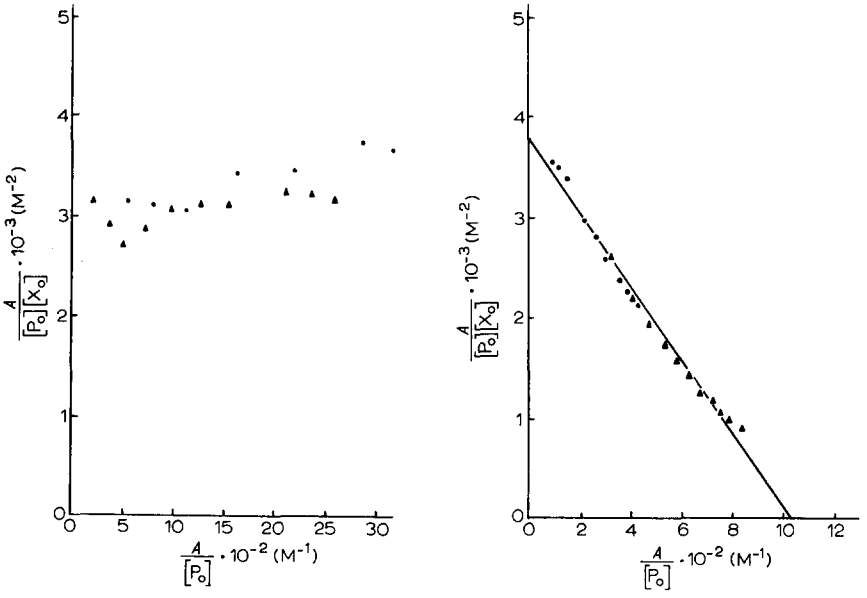


Fig. 2. Scatchard plot of the interaction of *N*-methylnicotinamide chloride with the sulfur-substituted (▲) and the sulfur-free (●) form of rhodanese. Rhodanese was tested at a concentration of $1.5 \cdot 10^{-4}$ M (▲) and $1.95 \cdot 10^{-4}$ M (●) in a 0.20 M Tris buffer, pH = 7.9 (HCl) with the molar concentration of *N*-methylnicotinamide chloride varying from 0.07—0.82 M. *A* represents the corrected absorbance of the pure complex; $[P_0]$ is the constant total concentration of donor; $[X_0]$ is the total concentration of the acceptor.

Fig. 3. Scatchard plot of the interaction of *N*-methylnicotinamide chloride with *N*-acetyl-L-tryptophanamide. The two levels of donor concentration used were $1.42 \cdot 10^{-3}$ M (▲) and $1.94 \cdot 10^{-3}$ M (●) in a 0.20 M Tris buffer, pH = 7.9 (HCl). See Fig. 2 for an explanation of the symbols on the ordinate and abscissa.

A is the absorbance at 350 nm; $[P_0]$ is the protein concentration, considered constant here; and $[X_0]$ is the concentration of *N*-methylnicotinamide chloride.

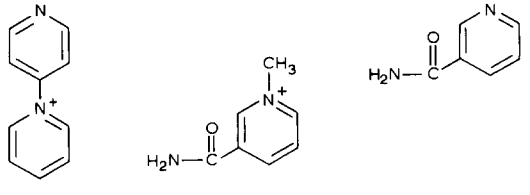
The slope is the negative of the association constant, k, between the donor and acceptor and the intercept on the abscissa gives the extinction coefficient of the charge transfer complex directly.

Deranleau and Schwyzer [11] examined several model compounds as donors and listed their formation constants with *N*-methylnicotinamide chloride and the molar extinction coefficients of these complexes (Table I).

To illustrate that this titration was valid, two independent experiments were performed with the model compound *N*-acetyl-L-tryptophanamide. This derivative was dissolved in 0.20 M Tris, pH 7.9 (HCl) to the concentrations of $1.42 \cdot 10^{-3}$ M and $1.94 \cdot 10^{-3}$ M. Omitting the column chromatography step, the same experimental procedure and data analysis was followed as above. The results of this experiment are shown in Fig. 3. An analysis of this plot by a linear least squares computer program yielded an extinction coefficient of 1035 M^{-1} cm^{-1} for the complex at 350 nm and an association constant of 3.6 M^{-1}. This binding constant corresponds well with the formation constants of this derivative with other indole compounds (Table I).

Discussion

We have tested the proposal that NPP (I) interacts with rhodanese via a charge transfer interaction by using *N*-methylnicotinamide chloride (II) and nicotinamide (III).



I II III

Scheme 1. I, *N*-1-(4-pyridyl)pyridinium chloride; II, *N*-methylnicotinamide chloride; III, nicotinamide.

Examination of the NPP structure (I) leads to three possible nonexclusive modes by which this compound could interact with proteins: the charge transfer interaction, an interaction with a negative charge on the protein, and/or interaction of the aromatic portion of NPP with an apolar site on the protein.

The compound *N*-methylnicotinamide chloride (II) can interact by both the charge transfer and ionic modes while the apolarity is reduced compared with NPP. In addition, the charge transfer interaction involving *N*-methylnicotinamide chloride is characterized by a charge transfer absorbtion band [10]. Nicotinamide (III) on the other hand does not have the ability to interact ionically but should still be able to bind via apolar interactions.

The inhibition studies using *N*-methylnicotinamide chloride indicate that this compound, like NPP is a competitive inhibitor of rhodanese with respect to thiosulfate. Nicotinamide also exhibited competitive inhibition with respect to thiosulfate, but at a much lower level as compared with *N*-methylnicotinamide

chloride and NPP. Hydrophobic interactions are thus eliminated as a major binding force but remain as a mode of stabilization since it has been reported that the active site is quite apolar [12].

Fig. 2 illustrates the data for the sulfur-substituted and sulfur-free forms of the enzyme. The Scatchard plots for rhodanese indicate no charge transfer interaction with *N*-methylnicotinamide chloride independent of the form of the enzyme. It is interesting that *N*-methylnicotinamide chloride has been used to identify solvent-accessible tryptophanyl residues in chicken egg-white lysozyme [10] and mouse 2.5 S nerve growth factor [13]. The latter study also illustrated the case of the absence of charge transfer as a collection of data points in a Scatchard plot very similar to those observed here. There are two requirements for the charge transfer interaction [13]:

1. The method is geometrically specific in that the planar ring of the indole moiety must be available for a face-to-face interaction with the ring of the positive pyridinium ion.

2. Space within the tertiary structure of the protein must be provided for the probe.

Therefore, although the data here does not permit a full assessment of the absence of an active site tryptophan it appears, at least, that the enzyme presents steric constraints to the formation of a charge transfer complex with the compounds tested to date.

The NPP structure (I) is approximately the "sum" of the individual *N*-methylnicotinamide chloride and nicotinamide structures and the association constants in Table I can be used to derive the free energy of interaction (ΔG°) for each of the tested compounds. NPP binds with a $\Delta G^\circ = -3.18$ kcal/mol. The sum of the ΔG° values for *N*-methylnicotinamide chloride and nicotinamide is -3.00 kcal/mol. The discrepancy in these ΔG° values may be due in part to the absence of the amide groups in NPP. Thus the binding of NPP to rhodanese can formally be accounted for by independent contributions from the two aromatic rings.

We conclude on the basis of these results that the binding of the pyridinium compounds tested so far is due mainly to an ionic interaction with accessory apolar stabilization. It is interesting that the parallel of the effectiveness of inhibitors with respect to the properties of the compounds previously tested [4] might be expected to hold for apolar as well as for charge transfer interactions. It is tempting to speculate that the enzyme anionic center is the ionized active site sulfhydryl group [14] previously shown to be essential [4].

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